From CENTER FOR INFECTIOUS MEDICINE,
DEPARTMENT OF MEDICINE
Karolinska Institutet, Stockholm, Sweden

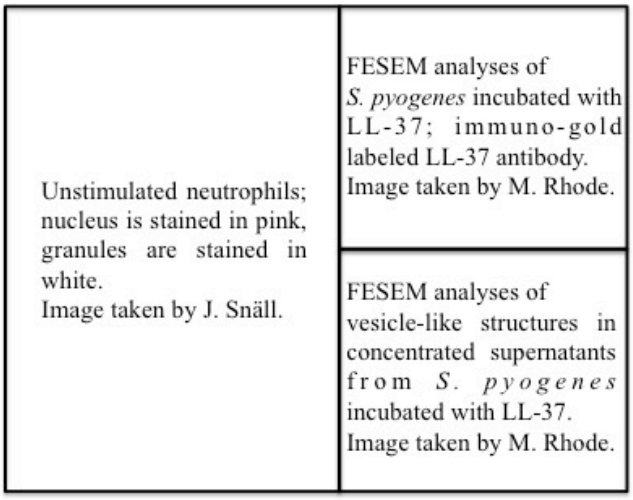
# NEUTROPHIL INTERACTIONS WITH *STREPTOCOCCUS PYOGENES* AND *STAPHYLOCOCCUS AUREUS*

Julia Uhlmann


Karolinska Institutet

Stockholm 2017

Front cover:





Published by Karolinska Institutet.
Printed by E-Print AB 2017

ISBN 978-91-7676-581-4

# Neutrophil interactions with *Streptococcus pyogenes* and *Staphylococcus aureus*

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Julia Uhlmann**

*Principal Supervisor:*
Prof. Anna Norrby-Teglund
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

*Co-supervisor(s):*
Ph.D. Linda Jervelius
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Assoc. Prof. Peter Bergman
Karolinska Institutet
Department of Medicine and
Laboratory Medicine
Center for Infectious Medicine and
Clinical Microbiology

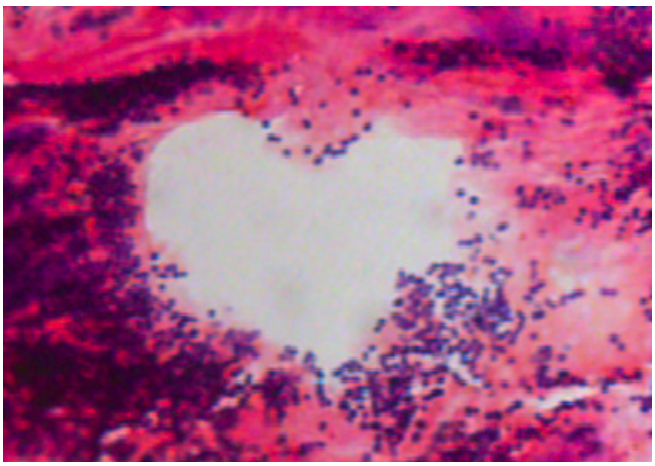
Dr. rer. nat. Nikolai Siemens
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

*Opponent:*
Prof. Victor Nizet
University of California, San Diego
Department of Pediatrics
Division of Host-Microbe System
and Therapeutics

*Examination Board:*
Prof. Anna Färnert
Karolinska Institutet
Department of Medicine, Solna
Division of Infectious Disease

Prof. Johan Bylund
University of Gothenburg
Department of Odontology

Prof. Annelie Brauner
Karolinska Institutet
Department of Microbiology, Tumor
and Cell Biology



Gram-stained patient biopsy. Image taken by B. Chakrakodi

**To my mum**

# ABSTRACT

*Streptococcus pyogenes* and *Staphylococcus aureus* are Gram-positive bacteria that share many features, including clinical presentations and pathogenic mechanisms, and yet, they still have unique properties. They both cause diseases ranging from uncomplicated infections to severe invasive diseases, such as sepsis and toxic shock syndrome, as well as severe tissue infections including necrotizing fasciitis and necrotizing pneumonia. While *S. pyogenes* is susceptible to penicillin, the public health concern regarding staphylococcal infections is enhanced by the increasing prevalence of methicillin-resistant *S. aureus*. Neutrophils have a central role as the first line of defense against bacteria, by killing invaders through phagocytosis, neutrophil extracellular trap formation or degranulation. Neutrophil granules contain a variety of proteins that contribute to antimicrobial defense. However, degranulation and release of these proteolytic and inflammatory factors in the tissue milieu can also be harmful to the host. This thesis project explored interactions between neutrophils and both Gram-positive species, with a particular focus on neutrophils as potential contributors to the pathogenesis of these infections.

In **paper I** we investigated the neutrophil response towards streptococcal factors secreted by different clinical isolates. We identified phosphoglycerate kinase (PGK), a moonlighting protein with glycolytic function, as a novel, potent neutrophil activator with the ability to trigger degranulation. PGK was found to be susceptible to proteolytic degradation by SpeB and consequently SpeB-negative strains elicited stronger neutrophil responses. This finding is of interest, as hypervirulent SpeB-negative strains are associated with invasive streptococcal infections.

In **paper II** we studied the effect of sub-inhibitory concentrations of LL-37 on *S. pyogenes*, as these concentrations have been reported to alter virulence gene expression. We showed that LL-37 induced the release of extracellular vesicle-like structures, which contained several virulence factors with immunostimulatory properties. This is the first report of vesicle-like structures release by *S. pyogenes* in response to LL-37. The presence of virulence factors in these vesicles and the pro-inflammatory effect towards neutrophils implicates a potential role for LL-37 in *S. pyogenes* pathogenesis.

In **paper III** we focused on bi-component leukocidins from *S. aureus* and their effect on neutrophil degranulation. PVL and LukED had a dose-dependent effect on cytotoxicity. However, neutrophil degranulation showed a different pattern. While PVL triggered even at sub-lytic concentrations neutrophil granule exocytosis, this event was only seen when lytic concentrations of LukED were used. Determination of the whole neutrophil secretomes triggered by lytic and sub-lytic concentrations of PVL and LukED revealed significantly different response profiles. This study demonstrates that neutrophil activation and degranulation in response to *S. aureus* pore-forming toxins depends on both the type and concentration of toxin. PVL was found to be a potent activator even at sublytic concentrations.

In summary, we identified the streptococcal factor PGK, as well as the pore-forming toxins from *S. aureus* as potent triggers of neutrophil activation and degranulation. In addition, we show that the antimicrobial peptide LL-37 induces the release of pro-inflammatory extracellular vesicle-like structures from the surface of *S. pyogenes*. Together, these studies demonstrate specific neutrophil responses triggered by Gram-positive bacterial virulence factors, which is in line with a likely contribution to disease pathogenesis.

# LIST OF SCIENTIFIC PAPERS

This thesis is based on two publications and one manuscript. The individual papers are referred to by roman numerals:

I. **Uhlmann J**, Siemens N, Kai-Larsen Y, Fiedler T, Bergman P, Johansson L, Norrby-Teglund A. Phosphoglycerate kinase – a novel streptococcal factor involved in neutrophil activation and degranulation. *Journal of Infectious Diseases (2016) 214: 1876-83.*

II. **Uhlmann J**, Rohde M, Siemens N, Kreikemeyer B, Bergman P, Johansson L, Norrby-Teglund A. LL-37 triggers formation of *Streptococcus pyogenes* extracellular vesicle-like structures with immune stimulatory properties. *Journal of Innate Immunity (2016) 8: 243-57*

III. **Uhlmann J**, Snäll J, Wajima T, Mairpady Shambat S, Vandenesch F, Bergman P, Siemens N, Norrby-Teglund A. Dissection of neutrophil cytotoxicity and exocytosis caused by *Staphylococcus aureus* leukotoxins. *Manuscript*.

# LIST OF ADDITIONAL PAPERS, NOT INCLUDED IN THE THESIS

- Snäll J, Linnér A, **Uhlmann J**, Siemens N, Ibold H, Janos M, Linder A, Kreikemeyer B, Herwald H, Johansson L, Norrby-Teglund A. Differential neutrophil responses to bacterial stimuli: Streptococcal strains are potent inducers of heparin-binding protein and resistin-release. *Scientific Report (2016) 6:21288. doi: 10.1038/srep21288*

- Beutler N, Hauka S, Niepel A, Kowalewski DJ, **Uhlmann J**, Ghanem E, Erkelenz S, Wiek C, Hanenberg H, Schaal H, Stevanović S, Springer S, Momburg F, Hengel H, Halenius A. A natural tapasin isoform lacking exon 3 modifies peptide loading complex function. *European Journal of Immunology (2013) 43(6):1459-69. doi: 10.1002/eji.201242725.*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AMP | Antimicrobial peptide |
| APC | Antigen presenting cell |
| BCL | Bi-component leucocidin |
| BCR | B cell receptor |
| BPI | Bactericidal permeability-increasing protein |
| C4BP | C4b binding protein |
| CA-MRSA | Community-acquired methicillin-resistan S. aureus |
| CAP1 | Adenylyl cyclase-associated protein 1 |
| CFU | Colony forming unit |
| CovRS | Control of virulence regulatory sensor kinase |
| CTL | Cytotoxic T lymphocyte |
| DAMP | Danger-associated molecular pattern |
| DC | Dendritic cell |
| FCS | Fetal bovine serum |
| Fn | Fibronectin |
| G-CSF | Granulocyte-colony stimulatory factor |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GAS | Group A Streptococcus |
| GRAB | G-related α2-macroglobulin-binding protein |
| $H_2O_2$ | Hydrogen peroxidase |
| HA-MRSA | Hospital-acquired methicillin-resistan S. aureus |
| HBP | Heparing binding protein |
| HBSS | Hank`s balanced salt solution |
| HDP | Host defense peptide |
| HlgAB/HlgCB | Gamma-hemolysin |
| HOCl | Hypochlorous acid |
| ICAM | Intracellular adhesion molecule |
| IFN | Interferon |
| IL | Interleukin |
| LDH | Lactate dehydrogenase |
| LFA-1 | Lymphocyte function associated antigen-1 |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| $LTB_4$ | Leukotriene B4 |

| | |
|---|---|
| Mac-1 | Macrophage-1 antigen |
| MBC | Minimal bactericidal concentration |
| MHC | Major histocompatibility complex |
| MIC | Minimal inhibitory concentration |
| MMP9 | Matrix metallopeptidase 9 |
| MPO | Myeloperoxidase |
| MRSA | Methicillin-resistant S. aureus |
| MSCRAMMs | Microbial surface components recognizing adhesive matrix molecules |
| MSSA | Methicillin-susceptible S. aureus |
| MV | Membrane vesicle |
| NET | Neutrophil extracellular trap |
| NF | Necrotizing fasciitis |
| NGAL | Neutrophil gelatinase-associated lipocalin |
| NK | Natural killer (cell) |
| OD | Optical density |
| $OH^{\bullet}$ | Hydroxyl radical |
| OMV | Outer membrane vesicle |
| p38MAPK | p38 mitogen-activated protein kinase |
| PAF | Platelet activation factor |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PGK | Phosphoglycerate kinase |
| PMN | Polymorphonuclear neutrophil |
| PRR | Pattern recognition receptor |
| PVL | Panton-Valentine Leukocidin |
| RELM | resistin-like molecule |
| ROS | reactive oxygen species |
| Sag | Superantigen |
| SIC | Serum inhibitor of complement |
| Ska | Streptokinase |
| SLO | Streptolysin O |
| SLS | Streptolysin S |
| SmeZ | Streptococcal mitogenic exotoxin |
| SOF | Serum opacity factor |
| Spe | Streptococcal pyrogenic exotoxin |

| | |
|---|---|
| SSA | Streptococcal superantigen |
| STSS | Streptococcal toxic shock syndrome |
| TCR | T cell receptor |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| VCAM | Vascular cell adhesion molecule |

# BACKGROUND

## 1.1 THE IMMUNE SYSTEM – AN OVERVIEW

Our body is protected by our immune system, which consists of different cells, tissues and organs and can be divided into innate and adaptive immunity (**Figure 1**) [1]. Innate immunity is characterized by the immediate and unspecific response to a microbial assault. In contrast, the adaptive immune response takes more time to become activated, but is very specific. Additionally, the adaptive arm of the immune response is endowed with an immunological memory of specific invaders.

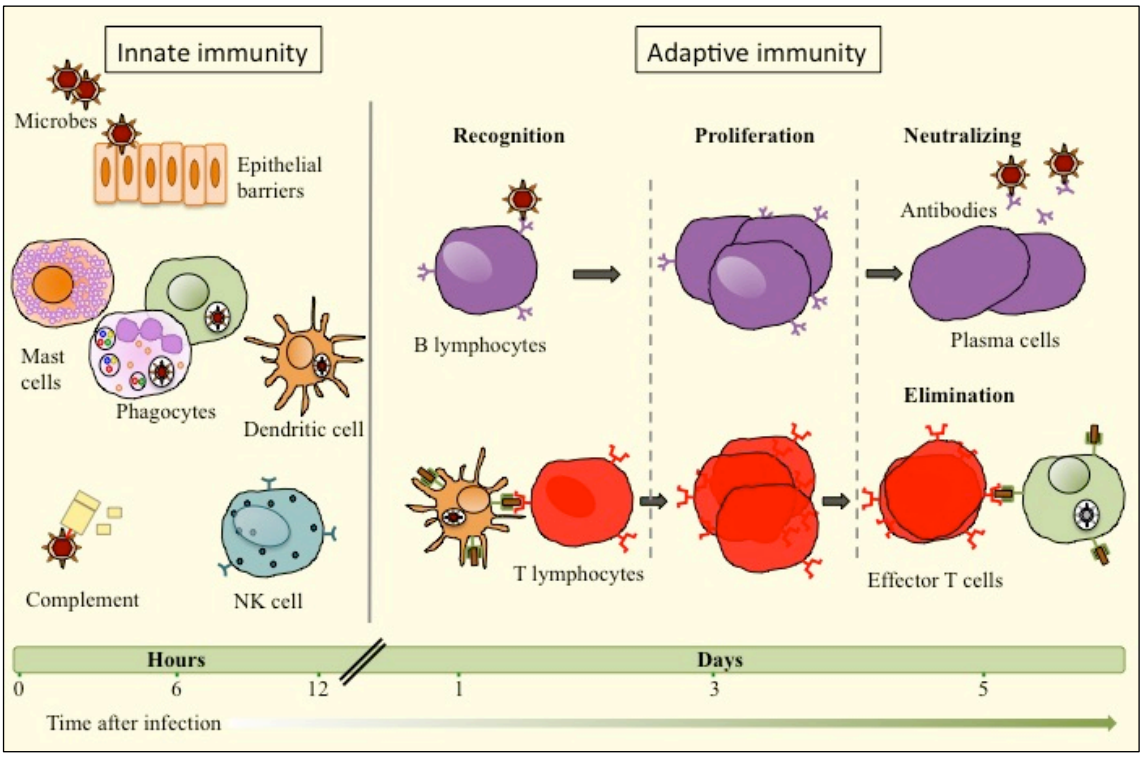



**Figure 1. Principal mechanisms of innate and adaptive immunity.** Innate immunity provides initial defense against invaders, such as epithelial barriers, different cells, e.g. phagocytes, and the complement system. The adaptive immunity develops later and consists of B and T lymphocytes. The recognition of microbes is mediated through specific receptors, following proliferation of the lymphocytes. Antibodies produced by plasma cell bind to microbes and label them for destruction by phagocytes. Effector T cells can eliminate infected cells by inducing apoptosis. Adapted and modified from [2].

## Adaptive Immunity

T- and B-lymphocytes are the major players in adaptive immune response (**Figure 1**) [1]. Both are produced in the bone marrow and have a diverse repertoire of receptors, either T cell receptors (TCRs) or B cell receptors (BCRs), which can recognize almost any intruder. While T cells recognize specific peptides derived from protein antigens, which are presented by infected cells via MHC class I or class II molecules, B-cells themselves are able to recognize any organic antigen without presentation.

T cells mature in the thymus where they differentiate into two different subsets. Depending on the type of glycoprotein (also known as co-receptors), they express on their surface, they are called either $CD4^+$ or $CD8^+$ T cells. $CD4^+$ T cells, also referred to as T helper ($T_H$) cells, assist other immune cells during infection. They recognize peptides, degraded from exogenous antigens that are presented by professional phagocytes (APCs) like Dendritic Cells (DCs), macrophages and B-cells via MHC class II. $CD8^+$, or cytotoxic T lymphocytes (CTLs), recognize peptides degraded from exogenous and endogenous antigens presented via MHC class I on any nucleated cell [3]. If a cell is infected, CTLs can kill it by inducing apoptosis.

B cells mature in the bone marrow and can produce large amounts of antibodies. These antibodies can help to neutralize virulence factors and to eliminate invaders by binding to antigens. This either prevents entry into and the subsequent infection of cells and/or will mark the pathogen for destruction by phagocytes (opsonization).

## Innate Immunity

It takes some time for the adaptive immune response to produce antibodies and/or increase the numbers of involved cells (by proliferation or clonal expansion). The innate immune system however reacts immediately upon the detection of foreign structures and is the first line of our defense against foreign bodies. It consists of a physical barrier – the skin and mucosa – that protects us from invading pathogens [4]. Once this barrier is breached there are professional phagocytes, such as macrophages, DCs and neutrophils, natural killer (NK) cells, antimicrobial peptides (AMPs), cytokines and components of the complement system present to face and neutralize these invaders [4]. When it comes to bacterial infections, neutrophils play a crucial role as they are amongst the first immune cells recruited to the site of infection (**Figure 1**).

## 1.2 NEUTROPHILS AND THEIR ROLE IN THE HOST DEFENSE AGAINST PATHOGENS

### 1.2.1 Neutrophils

Neutrophils are part of the innate immune system and belong to the group of granular leukocytes (granulocytes), as determined by the presence of different granules in their cytoplasm. In contrast, lymphocytes and monocytes are referred to as non-granular leukocytes. The shape of the nucleus, which is "folded" into three segments, has given the granulocytes the name polymorphonuclear (PMN) cells.

Neutrophils are derived from the bone marrow and make up around 70% of our circulating leukocytes. Every day, up to $0.5\text{-}1x10^{11}$ neutrophils are produced in the bone marrow [5] and this is controlled by granulocyte colony stimulatory factor (G-CSF) [6]. During inflammation or in response to the increased apoptosis of neutrophils, the rate of neutrophil production can also be augmented by IL-17 and other cytokines released by $T_H$ cells [7]. Neutrophils are generally thought to be short-lived cells, with a half-life of only 7h in circulation [8]; however, recently, it has been shown by Pillay *et al*. that circulating neutrophils can have a lifespan of around 5.4 days [9]. An explanation for this discrepancy in neutrophil lifespan could be a result of methodological problems in the labeling of neutrophils, as suggested by Tak *et al*. [10]. On the other hand, more and more data is emerging regarding additional neutrophil functions aside from their roles in the innate immune response and pathogen killing, e.g. modulation of B and T cell activation [11], which supports the argument that neutrophils have a longer lifespan.

### 1.2.2 Chemotaxis, priming and activation

Neutrophils circulate in the blood and are "on call", waiting for chemokine signals from infected tissue. If an invader is detected, neutrophils leave the blood stream and migrate into the targeted tissue, which involves different steps: tethering, rolling, adhesion, crawling and transmigration (**Figure 2**) [11].

Sentinel cells in tissue, such as macrophages, mast cells or DCs, release cytokines in response to pathogens or other danger signals. This induces changes in the endothelial cells present in the nearby blood vessels. Besides this indirect activation, endothelial cells

can also be activated by direct recognition of pathogens through pattern recognition receptors (PRRs).

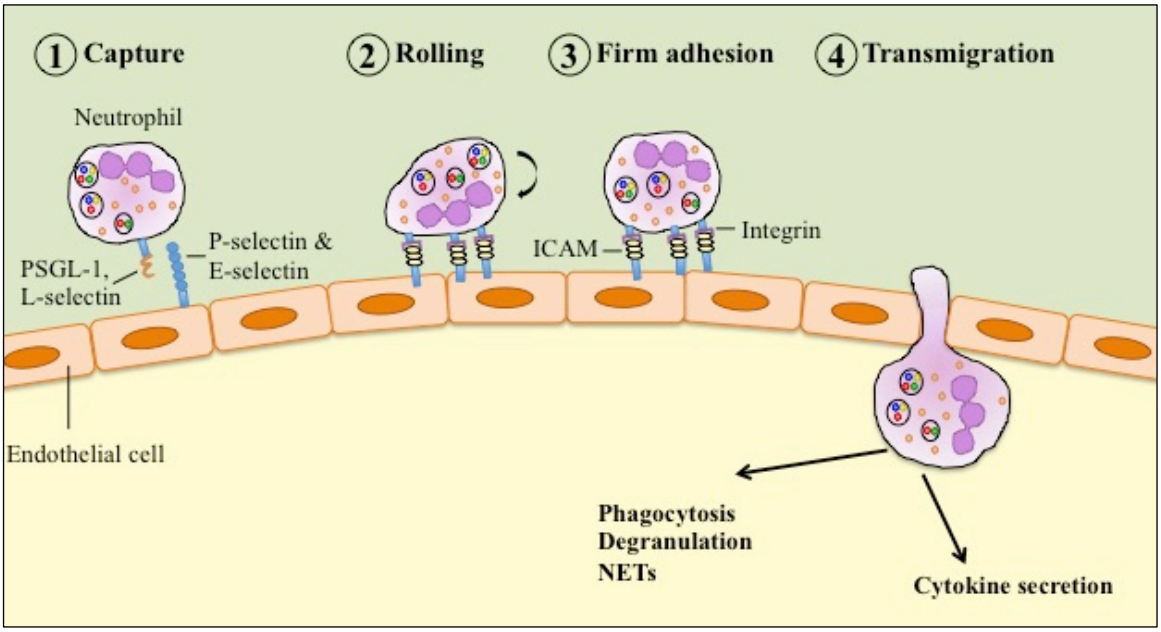



**Figure 2. Neutrophil recruitment to sites of inflammation.** 1) Circulating neutrophils are captured by selectins (P and E), which are expressed on the surface of stimulated endothelial cells, followed by selectin-mediated rolling (2). Integrin-integrin interactions result in firm adhesion (3) and final transmigration (4) through the endothelium to the site of infection. Adapted and modified from [12].

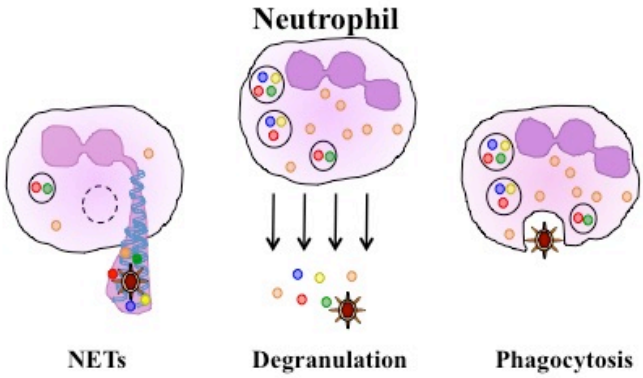
As a consequence of either indirect or direct recognition of pathogens, endothelial cells up-regulate different selectins (P- and E-selectin). Neutrophils express the complementary ligand P-selectin glycoprotein 1 (PSGL-1) on their surface, which interacts with the epithelial selectins. Through this interaction and the constant breaking and formation of new bonds between the selectins and neutrophils, the neutrophil speed slows down (tethering). Once the neutrophil speed is reduced (rolling), interactions between the integrins ICAM-1 and -2 (intercellular adhesion molecule-1; 2) present on endothelial cells with LFA-1 (lymphocyte function associated antigen-1) and Mac-1 (macrophage-1 antigen) on neutrophils, lead to closer contact (adhesion) (**Figure 2**).

Pro-inflammatory cytokines like TNFα (tumor necrosis factor alpha) and IL-1β (interleukin-1 beta) produced by sentinel cells and chemokines released from the endothelium cause priming of the neutrophil, which is required for later activation [13, 14]. Next, cytoskeleton rearrangement occurs; the neutrophil flattens and migrates through the endothelium (transmigration). Once on the other side of the endothelium, inside the tissue, neutrophils follow the signals from the sentinel cells and signals from

the “battle field” to the site of infection and are attracted by factors such as IL-8, leukotriene B4 ($LTB_4$), platelet activation factor (PAF), complement fraction C5a, and bacteria-derived fMLPs, amongst others (**Figure 2**) [15, 16].

### 1.2.3 Bactericidal effector mechanisms

Neutrophils have an important role in fighting pathogens. They are produced in high numbers and if an invader has been detected they are ready to leave the blood and migrate to the site of infection. In order to fight pathogens, neutrophils are equipped with a number of different killing mechanisms, such as the ability to form extracellular neutrophil traps (NETs; which are comparable with spider webs), phagocytosis of the invader and subsequent killing, and the release of preformed antimicrobial components that are stored in granules (degranulation) (**Figure 3**).




NETs = neutrophil extracellular traps

**Figure 3. Neutrophil killing mechanisms.** Adapted and modified from **[11].**

### 1.2.3.1 Phagocytosis

Neutrophils are professional phagocytes that recognize pathogens, engulf them and destroy them within the phagosome. This phagocytosis is very efficient and the internalization of particles occurs in less than 20 seconds [17]. In contrast, macrophages require several minutes for a similar target [18]. The initial step is the recognition of the target. For this purpose, neutrophils express different types of Pattern-recognition receptors (PRRs), e.g. Toll-like receptors (TLRs), which can directly recognize pathogens including bacteria, viruses and fungi by specific pathogen-associated- molecular patterns (PAMPs). Examples of such associations between PAMPs and their corresponding TLRs, (shown in brackets) include: lipopolysaccharide (LPS) from Gram-negative bacteria [TLR4], lipoteichoic acid (LTA) from Gram-positive bacteria [TLR2], bacterial DNA [TLR9] or double-stranded RNA from viruses [TLR8] [19]. In addition, indirect recognition, which is greatly facilitated by opsonized pathogens coated with antibodies or complement components, is performed by Fc and complement receptors [20].

After recognition and receptor binding, the neutrophil membrane extends around the pathogen, resulting in engulfment and uptake and the trapping of pathogen in the phagosome. During phagosome maturation, their fusion with endosomes and different granules (more details given in 1.2.3.3 Degranulation) induces the release of factors like lysozyme, antimicrobial peptides (AMPs), proteases, and MPO (myeloperoxidase) into the phagosome, which results in the efficient killing of the engulfed pathogen [21]. In addition, oxidative metabolism is up-regulated and provides, through the membrane bound NADPH oxidase, the reactive oxygen species (ROS) hydrogen peroxidase ($H_2O_2$), hydroxyl radical ($OH^{\bullet}$) and hypochlorous acid (HOCl), all of which further facilitate the successful killing of pathogens in the phagosome [22-25].

### 1.2.3.2 Neutrophil Extracellular Traps (NETs)

In 2004, a new mechanism of neutrophil killing was described by Brinkmann *et al*., where the authors showed that activated neutrophils form neutrophil extracellular traps (NETs) consisting of chromatin DNA, histones, enzymes, AMPs like LL-37 and other granule proteins [26]. The released DNA builds a backbone for the granule derived components and the formation of NETs occurs 10 minutes after activation [26, 27]. Formation of NETs is an additional form of neutrophil mediated cell death, called NETosis. Variations

exist between the classical form, called suicidal NETosis and a more recently described vital NETosis [28]. Suicidal NETosis is characterized by the rupture of the outer membrane, followed by the release of mature NETs and can be induced by, for instance PMA or IL-8 [26]. In contrast, neutrophils undergoing vital NETosis preserve an intact membrane and maintain their functions [28].

NETs are able to trap bacteria and prevent them from spreading in the tissue. In addition, the presence of high concentrations of serine protease in NETs can kill these trapped bacteria either directly or indirectly by cleaving the human cathelicidin antimicrobial peptide (hCAP-18) which releases the antimicrobial peptide LL-37 from its C-terminal end [26]. It has been documented that this new mechanism of neutrophil killing is relevant for the targeting of both Gram-negative, as well as Gram-positive bacteria [26].

### 1.2.3.3 Degranulation

#### Granule types

Neutrophils develop in the bone marrow and go through different maturation stages: myeloblast, promyeloblast, myelocyte, metamyelocyte, band cell and finally a mature neutrophil [29]. During these stages, also called granulopoiesis, they produce different types of granules in which preformed effector molecules are subsequently stored [30, 31]. Neutrophils maintain their dividing potential until the metamyelocyte stage whereas granule maturation ends with the mature neutrophil (PMN) (**Figure 4**) [32, 33].

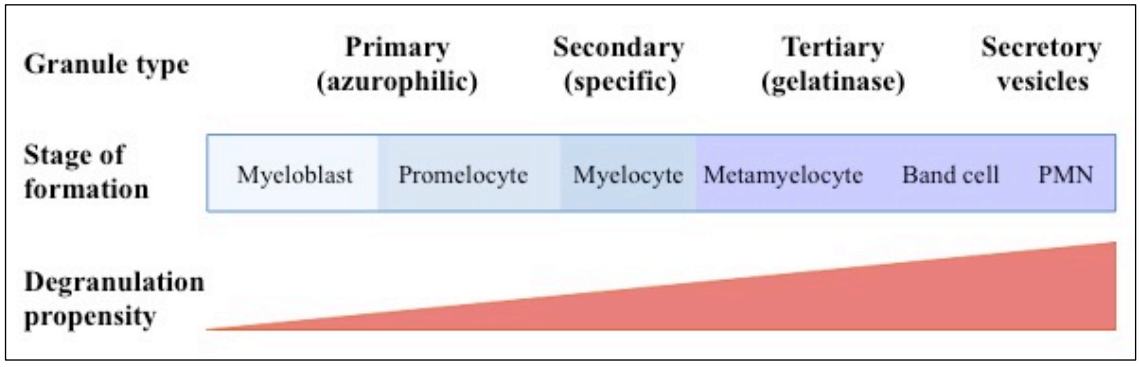



**Figure 4. Granule development.** Neutrophils develop four types of granules: primary or azurophilic, secondary or specific, and tertiary or gelatinase, and secretory vesicles. The development of these granules is reverse to the degranulation propensity. Adapted and modified from [12].

When full neutrophil maturation has concluded, which takes approximately 2 weeks [34], all 4 granule types have been formed, namely azurophilic granules, also called primary granules, specific granules (secondary), gelatinase granules (tertiary) and secretory vesicles. The granule/vesicle content is dependent on the development stage of the neutrophil and the synthesized proteins are included from promyeloblasts to the band cell stage. Factors stored in these granules include antimicrobial components, soluble factors of inflammation, components of the respiratory burst oxidase, and adhesion molecules (**Table 1**) (**Figure 4**) [35, 36].

**Azurophilic granules** have high amounts of myeloperoxidase (MPO) and are also characterized by their peroxidase positivity in contrast to the other granules that are peroxidase negative [37, 38]. Other important components of azurophilic granules include different acidic hydrolases, serine proteases, defensins and bactericidal permeability-increasing protein (BPI) [39, 40]. These granules play an important role in killing pathogens through their fusion with the phagosome and release of azurophilic components (**Table 1**) [41].

**Table 1: Content of neutrophil granules.** Modified and adapted from [42]**.**

| | **Azurophilic (primary) granules** | **Specific (secondary) granules** | **Gelatinase (tertiary) granules** | **Secretory vesicles** |
|---|---|---|---|---|
| Proteases | Elastase<br>Cathepsin G<br>Proteinase-3 | Collagenase (MMP-8) | Gelatinase (MMP-9)<br>Arginase 1<br>Leukolysin (MMP-25) | Leukolysin (MMP-25)<br>Proteinase-3 |
| Anti-bacterial proteins | Myeloperoxidase (MPO)<br>Bactericidal/permeability-increasing protein (BPI)<br>Defensins<br>Lysozyme | hCAP-18<br>Neutrophil gelatinase-associated lipocalin<br>$B_{12}$ –binding protein<br>Lysozyme<br>Lactoferrin<br>Haptoglobolin<br>Pentrax 3<br>Prodefensin<br>$Gp91^{phox}/p22^{phox}$ | Lysozyme<br>$Gp91^{phox}/p22^{phox}$ | $Gp91^{phox}/p22^{phox}$ |
| Adhesion molecules | | CD11b/CD18<br>CD66<br>CD67 | CD11b/CD18<br>CD67 | CD11b/CD18<br>CD67 |
| Receptors | | uPAR<br>Laminin-R<br>Thrombospondin-R | | CD35 (CR1)<br>CD16<br>CD14<br>C1q-R<br>fMLPR |
| Other classes of functional proteins | Sialidase<br>Heparing binding protein (HBP)<br>β-Glucuronidase<br>Granulophysin (CD63)<br>Presenilin<br>Acid β-Glycerophosphosphatase<br>Acid mucopolysaccharide<br>α-1 Antitrypsin<br>α-Mannosidase<br>β-Glycerophosphosphatase<br>N-acetyl-β-glucosaminidase<br>Resistin * | Secreyted leukocyte peptidase inhibitor<br>Orosomucoid<br>β2-Microglobulin<br>Histaminase<br>Heparanase<br>CRISP3<br>SNAP-23<br>VAMP-2<br>Stomatin | β2-Microglobulin<br>CRISP3<br>SNAP-23<br>VAMP-2<br>Nramp1 | Plasma proteins (including albumin)<br>SNAP-23<br>VAMP-2<br>Nramp1<br>Alkaline phosphatase<br>DAF<br>CD10<br>CD13<br>Heparing binding protein (HBP) |

R = receptor; * = [43]

**Specific granules** contain proteins such as lactoferrin and NGAL (neutrophil gelatinase-associated lipocalin) [44] and other antimicrobial components. Upon mobilization they fuse with either the phagosome or they release their stored effector molecules by degranulation (**Table 1**) [42].

**Gelatinase granules** are rich in MMP9 (matrix metallopeptidase 9), gelatinase, and play an important role in the process by which neutrophils leave the blood stream through the provision of receptors and matrix-degrading enzymes (**Table 1**) [42].

**Secretory vesicles** contain plasma proteins and other distinct receptors on their membrane, which are important for interactions with the endothelium, monocytes, DCs and provide neutrophils with a wider repertoire for receiving inflammatory signal (**Table 1**) [42].

Release of different granules is not a random process, but rather a closely controlled, specific event. Whilst in the blood stream and until the neutrophils have reached the site of infection, granules only fuse with the extracellular membrane or the phagosome when needed [45]. Therefore secretory vesicles are mobilized first, to facilitate the contact between the neutrophils and the endothelium. These are followed by the gelatinase granules (during diapedesis), specific granules and finally the azurophilic granules (secretory → tertiary → secondary → primary) (**Figure 4**).

**Degranulation Mechanism**

The release of granule components in either the phagosome or into tissue is a tightly controlled receptor mediated mechanism which requires $Ca^{2+}$, ATP (adenosine triphosphate) and GTP (guanosine triphosphate) [46]. It involves granule recruitment, tethering and docking, priming and finally fusion and release of the granular proteins [46]. When neutrophils are at the site of infection and/or have engulfed pathogens, receptor recognition induces the remodeling of the actin cytoskeleton, which leads to the mobilization of granules in the cytoplasm to the outer membrane for degranulation, or for fusion of granules with the phagosome. Once the granules have reached the target membrane, they dock and fuse with the membrane, which in turn results in the exocytosis of granular components [46].

### 1.2.4 Neutrophil effector molecules

Neutrophils store pre-synthesized enzymes in granules and generate toxic chemicals for killing pathogens in the phagosome. However, killing of extracellular bacteria by exocytosis of granule components into the surrounding milieu, can also have harmful effects, such as inducing tissue injury (e.g. through proteases) or contributing to the disease by increasing vascular leakage (e.g. through HBP) and thereby further complicating severe inflammation (**Figure 5**) [47, 48]. In addition, the lifespan of circulating neutrophils is relatively short and "old" neutrophils undergo spontaneous apoptosis [49], which requires their subsequent removal by phagocyte efferocytosis to prevent lysis and release of granule proteins. Therefore, the recruitment of neutrophils needs to be controlled in relation to the level of the threat and removal of apoptotic neutrophils is important for maintaining tissue homeostasis [50].

In my thesis I focus on two neutrophil effector molecules – resistin and the antimicrobial peptide LL-37 - which will be described in more detail in the following section.

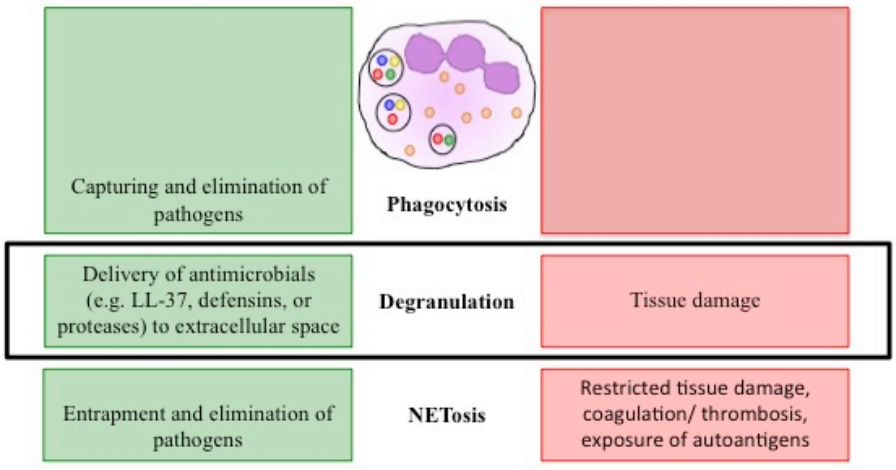



**Figure 5. Balancing act of neutrophil killing mechanisms.** My thesis is focused on the neutrophil degranulation and effects on the host (framed box); negative effects are shown in red and positive effects are shown in green. Adapted and modified from [51]**.**

### 1.2.4.1 Resistin

#### 1.2.4.1.1 Structure and source of resistin

Resistin, a 108 amino acid, 12.5 kDa peptide hormone, was first discovered in mice in 2001 and belongs to the resistin-like molecules (RELM) family [52-54]. The name "resistin" originates from observations in mice where resistin modulates insulin resistance [54].

In rodents, resistin is strongly detected in adipocytes as well as many other tissues and cells [55], whereas, in contrast, human adipocytes are minor producers. Resistin is mainly found in human monocytes and neutrophils [43, 56-58], but also in pancreatic islets [59], in placental tissue [60], as well as in the plasma [61].

Mouse and human resistin share only 59% homology at the amino acid level, which might explain the distinct functions that have been reported in different species. In the mouse, resistin is involved in obesity and type 2 diabetes mellitus [54], whereas in human, it is associated with pro-inflammatory conditions such as acute and chronic inflammation [43, 62-66]. Moreover, its role in insulin resistance and obesity in humans remains to be clarified [67-69].

#### 1.2.4.1.2 Resistin in inflammation

Human resistin contributes to pro-inflammatory conditions by inducing TNF-α and IL-12 production in macrophages [70] and TNF-α and IL-6 release in PBMCs and adipocytes [62, 71]. Activation of the transcription factor NF-κB appears to regulate the inflammatory properties of human resistin [70]. The receptor for resistin was only recently identified, where studies in humanized mice showed that resistin directly binds to CAP1 (adenylyl cyclase-associated protein 1), resulting in inflammatory responses [72].

In endothelial cells, resistin stimulates the expression of adhesions molecules (vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) through the p38MAPK (p38 mitogen-activated protein kinase) signaling pathway [73]. Similarly, resistin activates same pathway in platelets to increase P-selectin expression [74]. Furthermore, resistin is associated with up-regulation of P-selectin and fractalkine on monocytes [75].

Up-regulation of adhesion molecules and its role in inducing pro-inflammatory cytokines highlight the important role of resistin in inflammation. In patients with rheumatoid arthritis, a correlation with increased resistin levels was found [76, 77]. Additionally, our group has shown that there is a link between resistin levels and the severity of disease in severe sepsis and septic shock [43, 78].

### 1.2.4.2 The antimicrobial peptide LL-37

LL-37 belongs to the cathelicidins family that together with defensins constitutes the major classes of antimicrobial peptides (AMP) or host defense peptides (HDPs). Cathelicidin peptides are present in many species, suggesting they have an important immune role in many organisms [79]. These peptides exhibit antimicrobial activity against different types of pathogens, such as viruses, parasites and bacteria.

#### 1.2.4.2.1 Structure of LL-37

LL-37 is the only member of the cathelicidin-family found in humans and it was discovered simultaneously in 1995 by three different groups [80-82]. It was first named FALL-39 due to the 39 amino acid sequence at the N-terminus that started with FALL [80]. Later it was shown that the active form consists of 37 amino acid residues and therefore the peptide was named after the two leucines (LL) at the N-terminal end. LL-37 is an amphipathic α-helical peptide and is expressed as the precursor hCAP-18, which has a molecular weight of 18 kDa. It consists of a signal peptide, made up of the typical cathelin domain and an active peptide at the C-terminus (**Figure 6**). Once the peptide has reached its destination – either the cell membrane or the granules – the signal sequence is removed by cleavage and the proprotein is stored in an inactive form [83].

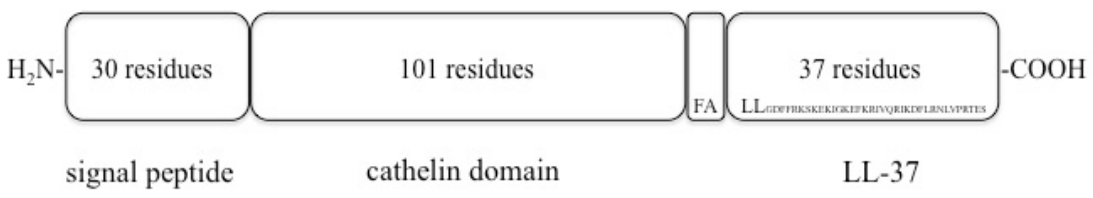



**Figure 6. The only human cathelicidin, hCAP18 and its C-terminal peptide LL-37**. Adapted and modified from [83]**.**

#### 1.2.4.2.2 Expression and regulation

Cathelicidins are a central component of the first line of defense against pathogens and are expressed in different cell types and tissues that are exposed to direct contact with the environment. The human cathelicidin LL-37 is found in different epithelial cells, for instance, the intestine, lung and skin but also in many immune cells including neutrophils, monocytes, macrophages, DCs, NK cells, lymphocytes and mast cells [84, 85]. In neutrophils LL-37 is stored in specific granules in its proform [86], whereas in keratinocytes the expression can be induced, e.g. during inflammatory disorders [87]. Only when neutrophil specific granules fuse with azurophilic granules or during NET formation with the release of the protease 3, can the inactive form be cleaved, producing active LL-37 peptide [88].

#### 1.2.4.2.3 Antimicrobial effect

LL-37 has broad antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as against fungi and viruses. The bactericidal action of LL-37 is exerted via the disruption of the bacterial membrane by the toroidal pore mechanism [89].

Bacterial membranes, in contrast to mammalian cells, are more negatively charged, due to the presence of anionic phospholipids and LPS in Gram-negative bacteria and teichoic acids in Gram-positive membranes [90]. This enables an electrostatic interaction between these negative components and the cationic properties of LL-37.

Through the formation of oligomers, LL-37 can reach the outer membrane of bacteria, where it binds in parallel to the bacterial surface. This allows the positively charged amino acids of LL-37 to interact with the head groups of the negatively charged phospholipids. As a result of this, the bacterial membrane bends and a toroidal hole is formed [91, 92]. This leads to the disruption of the bacterial membrane, loss of membrane potential, leakage of cellular components and subsequent killing of the bacteria [89, 91].

## 1.3 STREPTOCOCCUS PYOGENES AND STAPHYLOCOCCUS AUREUS

### 1.3.1 *Streptococcus pyogenes*

*Streptococcus pyogenes*, also known as Group A Streptococcus (GAS), is a Gram-positive bacterium, which lives under facultative anaerobic conditions. It grows in chains or pairs and induces beta-haemolysis. The ability of *S. pyogenes* to induce the production of pus gave it its name, which originates from the Greek pyo (pus)-genes (forming) and kokkos (berries). *S. pyogenes* is an exclusively human pathogen and can cause infections ranging from those that are mild to more severe and potentially life-threating infections.

#### 1.3.1.1 Non-invasive and invasive infections

*S. pyogenes* causes a range of uncomplicated skin and soft tissue infections, e.g., impetigo or erysipelas/cellulitis (**Table 2**). The most common, non-invasive *S. pyogenes* infections occur in the throat, i.e. tonsillitis and pharyngitis, with an estimated 616 million cases per year worldwide [93]. Penicillin is the antibiotic of choice for treatment against *S. pyogenes*, as it remains uniformly susceptible to this antibiotic [94]. However, recurrent throat infections can occur, which may be caused by the ability of *S. pyogenes* to hide inside pharyngeal epithelial cells after antibiotic treatment, as shown by Osterlund *et al*. [95].

**Table 2. Infections caused by *S. pyogenes***

| Superficial skin and throat infections | Invasive infections |
|---|---|
| Erysipelas<br>Impetigo<br>Pharyngitis<br>Scarlet fever<br>Tonsillitis | Bacteremia<br>Necrotizing fasciitis (NF)<br>Osteomyelitis<br>Pneumonia<br>Septic arthritis<br>Streptococcal toxic shock syndrome (STSS) |

*S. pyogenes* might also cause invasive infections, including bacteremia, pneumonia, necrotizing fasciitis (NF), sepsis and streptococcal toxic shock syndrome (STSS), which are associated with marked morbidity and mortality (**Table 2**) [93, 96]. Listed as number 9 on the list of global killer pathogens, *S. pyogenes* induces an estimated 500.000 deaths yearly [93]. Characteristics of these severe invasive infections include multiple organ failure, hypotension and shock, systemic toxicity, rapid necrosis of tissue and skin, and severe local pain [96].

In addition, *S. pyogenes* infections can lead to post-streptococcal sequelae such as rheumatic fever, rheumatic heart disease, glomerulonephritis, and arthritis, amongst others. Children and young adults with untreated pharyngitis can develop acute rheumatic fever (ARF), which in some cases can lead to rheumatic heart diseases and subsequent heart failure and death [97].

#### 1.3.1.2 Epidemiology

*Streptococcus pyogenes* isolates can be identified by three different serological and one genotypic classification systems:

- M serotyping
- T serotyping
- Serum opacity factor (SOF)
- *emm*-sequencing

The oldest method is M-typing, introduced in 1928 by Rebecca Lancefield [98]. This method is based on the surface-expressed M protein. In addition, serological typing can be based on another protein – the T-antigen [99] or alternatively, on the serum opacity factor [100]. Today, *emm*-sequencing is the most commonly used method, which is PCR based and allows the distinction of different M types strains as well as variants within an *emm*-type [101].

Some M types can be associated with clinical phenotypes, as they are overrepresented in these diseases, e.g. M1 and M3 types are commonly associated with invasive infections, such as STSS and NF (including M28), whereas M28 is the only type associated with puerperal sepsis, due to its virulence factor R28, which promotes adherence to vaginal

mucosa. In addition, M1 and M12 are associated with meningitis and M5 and M18 with epidemic ARF, which illustrate a few examples [102].

### 1.3.1.3 Virulence factors

*Streptococcus pyogenes* is an exclusively human pathogen, and it has adapted to its host through the expression of different virulence factors specific for humans (**Table 3**). Generally, surface virulence factors help the bacteria to initiate contact with host cells and to hide from the immune system, while secreted virulence factors assist in the dissemination into tissues and the generation of nutrients for the growth of the bacteria [103].

**Table 3. *S. pyogenes* virulence factors.** Modified and adapted from [103].

| **Cell-surface virulence factors** | | **Secreted virulence factors** | |
|---|---|---|---|
| Antiphagocytic | M protein<br>M-related proteins<br>Hyaluronic acid capsule<br>C5a peptidase | Spread through tissues | Hyaluronidase<br>Streptokinase<br>SpeB (cysteine protease)<br>DNases |
| Adherence and Internalization | Lipoteichoic acid<br>Fibronectin-binding proteins<br>M protein<br>Hyaluronic acid capsule<br>Protein F1 | Immune stimmulatory and immune evasion | Streptolysin O (SLO)<br>Streptolysin S (SLS)<br>Superantigens: SpeA, SpeC, SpeG, SpeM, SSA, SmeZ |

Spe (A, C, G, M) = streptococcal pyrogenic exotoxins; SSA = streptococcal superantigen; SmeZ = streptococcal mitogenic exotoxin

#### 1.3.1.3.1 Surface virulence factors

A number of important surface virulence factors exist, and amongst others, they include the capsule, different adhesion proteins and the M protein. The capsule consists of hyaluronic acid, which is similar to host hyaluronan and therefore a poor immunogen [104]. All these aforementioned virulence factors inhibit phagocytosis directly, or indirectly by interfering with opsonization by the complement system or through

antibodies. This antiphagocytic effect is also mediated through the M protein [105, 106], M like proteins [107, 108] and C5a peptidase (**Table 3**) [109].

One of the best-studied surface virulence factors is the M protein. I will use this molecule as an example of the different evasion strategies that are also employed by many other streptococcal surface proteins. The initial task of GAS is to attach and adhere to the host cells or tissue e.g. in skin infections, the M protein facilitates attachment to keratinocytes [110].

The binding of different components of the host extracellular matrix (ECM), such as collagen and fibronectin (Fn) facilitates the adhesion of the bacteria to host cells [111-113]. Other surface factors that can bind Fn are protein F1, protein F2, FbaA, FbaB, SfbI, SfbII and SOF [114].

The second important role of adhesins is to avoid recognition and elimination by the immune system. Opsonization and thereby phagocytosis can be inhibited by interfering with the complement cascade. The M protein binds to the C4b binding protein (C4BP) [115], which is a human complement inhibitor, and thus prevents opsonization and phagocytosis. Similar functions have been described for attaching Factor H and Factor H like proteins [106, 115, 116]. A passive method used by *S. pyogenes* to escape the complement is achieved by binding components from the blood, like albumin and fibrinogen which hides the bacterial surface from complement binding [117]. Other fibrinogen binding proteins include: Sfb1/PrtF1 and F2, M-like proteins and Mrp [118]

Surface bound M protein can be cleaved by the streptococcal cysteine protease SpeB. The soluble form can build complexes with fibrinogen, which in turn activates neutrophils resulting in degranulation and a subsequent increase in the inflammatory response, as well as increased capillary permeability and consequently vascular leakage [119]. In addition, the M protein can induce a massive T cell response, inflammation [120, 121] and it also plays a role in bacterial internalization and invasion [122, 123].

Most of the adhesion proteins are covalently anchored in the bacterial membrane by the typical LPxTz motif, in a similar way to the aforementioned proteins. *S. pyogenes* also expresses five anchorless adhesins that are involved in glycolytic processes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), α-enolase, phosphoglucerase mutase and triosephosphate isomerase [124].

#### 1.3.1.3.2 Secreted virulence factors

Characteristics of invasive infections consist of systemic toxicity and tissue destruction caused by secreted virulence factors [125]. Hyaluronidase is one of the enzymes which facilitates the degradation of hyaluronic acid in connective tissue, thereby facilitating the spread of infection [126]. Streptokinase (Ska) has an indirect influence on the spread of the infection through tissue by activating the host zymogen form plasminogen to plasmin. Active plasmin then dissolves the fibrin barriers therefore helping GAS to spread [103, 127]. Another important virulence factor is the cysteine protease SpeB (**Table 3**). This secreted factor is not only capable of degrading several host factors e.g. immunoglobulins [128], chemokines [129], fibrinogen [130, 131], plasminogen [132] and LL-37 [133, 134], but also several of its own factors such as Protein F1 [135], streptokinase [136] and Sda1 [137]. DNases, expressed by *S. pyogenes,* help to degrade the DNA backbone of NETs, thus impairing the killing mechanism [27, 138].

*S. pyogenes* exerts its systemic toxicity via the expression of different types of superantigens and two hemolysins. Streptococcal hemolysins, Streptolysin O and S (SLO and SLS), can form pores in the membranes of host erythrocytes [139, 140], neutrophils [103] and platelets [141]. SLO (oxygen labile) binds to host membranes as monomers which are rich in cholesterol [142], following oligomerazation thus causing the development of pores [139, 143]. It has been suggested that SLO is associated with invasive infections as its expression levels are higher in invasive *S. pyogenes* isolates compared to non-invasive isolates [144]. Neutrophil function was not only impaired through lysis, but sub-lytic concentrations of SLO early in infection suppressed the oxidative burst and NET formation [145]. SLO-triggered aggregation of neutrophils-platelets complexes is suggesting a role for SLO in vascular dysfunction in severe infections [146]. In addition, SLO can trigger an exaggerated neutrophil response, which results into the release of pro-inflammatory mediators [147]. SLS (serum soluble) is, in contrast to SLO, oxygen stable, but thermo-labile. Recently, the mechanism behind the lysis in erythrocytes has been identified; SLO disrupts the anion exchange protein, band 3, thereby initiating the influx of Cl, following lysis (**Table 3**) [140].

Superantigens (SAgs) can contribute to diseases such as STSS and NF by inducing a "cytokine storm" and therefore contributing to both shock and organ failure [148-150]. They can bind simultaneously as unprocessed, intact molecules to the variable β-chain of the TCR and MHC class II molecule on antigen presenting cells [151, 152]. This results

in substantial T cell activation, which is associated with an excessive release of pro-inflammatory cytokines, e.g. IL-1 and 2, TNF-α, TNF-β and INF-γ (**Table 3**) [151, 153].

### 1.3.2 *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive, catalase- and coagulase-positive bacterium and lives under facultative anaerobic conditions. *S. aureus* typically grows in grape-like clusters, and is part of the human flora of the skin and nasal mucosa. About 30% of the nasal flora is asymptomatic colonized by *S. aureus* [154, 155]. The name *aureus* originates from the golden pigmentation of the colonies (latin "aurum" for gold) and *Staphylococcus* from the Greek word for grape "staphylos" and kokkos "berry".

#### 1.3.2.1 Infections and epidemiology

With respect to *S. aureus* infections the increased development of antibiotic resistance, especially against penicillin, is of great concern. Methicillin-resistant *S. aureus* (MRSA) is resistant to penicillin and all other β-lactam antibiotics and causes the majority of hospital-acquired infections [156-158]. MRSA infections are associated with higher mortality and morbidity and are costly for the health system due to longer stays in hospital, when compared to methicillin-susceptible *S. aureus* (MSSA) [159]. The infections range from mild skin infections e.g. abscesses, to invasive and life-threatening infections such as pneumonia, sepsis, and infective endocarditis, amongst others [160]. However, people who are colonized by *S. aureus* are at greater risk of developing infections [161]. Between 2003 and 2005, around 400.000 *S. aureus* infections occurred each year in the USA, whereof 20.000 patients died in the hospital [162, 163].

*S. aureus* is recognized as major cause of hospital-acquired (HA) infections. However, in the last decade the separation between community-acquired (CA)- and HA-MRSA has become less defined, as CA-MRSA strains have infiltrated the hospital and replaced HA-MSRA strains [164, 165]. This infiltration and replacement suggest that some CA-MRSA strains are more aggressive in terms of virulence and transferability. CA-MRSA has remained more sensitive to antibiotic treatment due to the lack of multiple antibiotic resistances, apart from the penicillin resistance [159, 165].

### 1.3.2.2 Virulence factors

*S. aureus* expresses a wide range of secreted and surface bound virulence factors to enable survival in the host and to escape the immune response. Surface virulence factors like protein A (know from many biological assays by virtue of its affinity to the Fc-portion of antibodies), fibronectin-binding proteins, and clumping factors, amongst others, can promote adhesion to host cells and tissue, invasion and internalization and also help the bacterium to escape phagocytosis. Secreted virulence factors such as superantigens, cytolysins (e.g. alpha toxin, bi-component leucocidins (BCLs) such as PVL (Panton-Valentine Leukocidin) etc.), and exoenzymes play a major role in the outcome of infection as well [166, 167].

For the purpose of my thesis, the bi-component leucocidins (BCLs) are described in more detail below. I have focused on these as all BCLs have been shown to have a cytotoxic effect against several immune cells including human neutrophils [168].

#### 1.3.2.2.1 Bi-component pore-forming leucocidins

In human infections, four different *S. aureus* bi-component leucocidins have been identified; PVL, two forms of γ-hemolysin (HlgAB and HlgCB), LukED, and LukAB (also known as LukGH) (Table 4). Each leucocidin consists of two components named after their elution profile S (slow) and F (fast) [169]. The S subunit establishes the first contact with the host cell by recognizing a specific receptor. After the S subunit binds to the target cell receptor it recruits the F subunit [170], which is followed by dimerization and results in an oligomer structure. This oligomer consists of 4 S and 4 F subunits that form a β-barrel pore in the host membrane (**Figure 7**) [171, 172]. This characteristic β-barrel pore formation is also a feature of the cytotoxin α-toxin, however the α-toxin oligomerization only requires 7 components [173, 174]. While α-toxin is not toxic to neutrophils, all bi-component leucocidins are (**Table 4**).

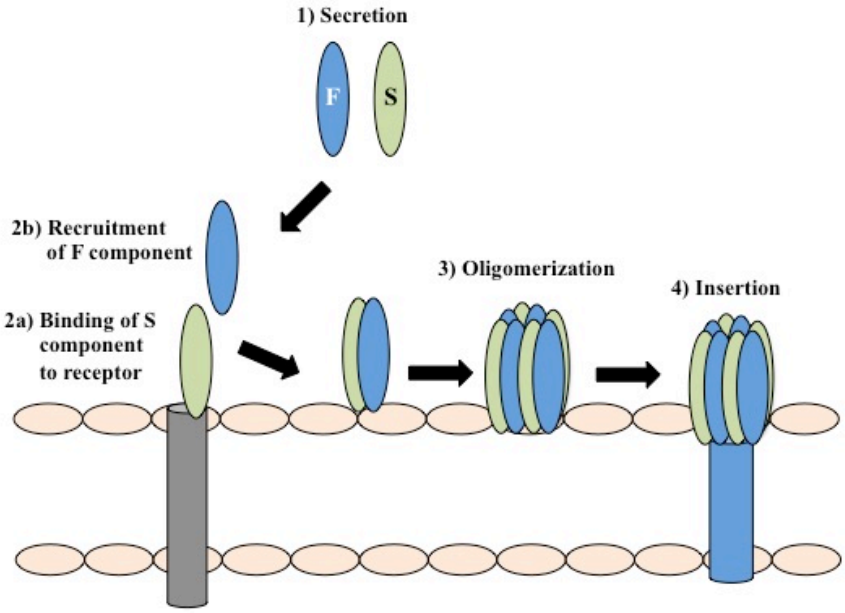



**Figure 7. Mechanism of pore formation of bi-component leucocidins (BCLs).** BCLs are composed of an S-subunit (S) and an F-subunit (F). After secretion (1), S binds to a specific receptor (grey) on the target cell (2a). Following recruitment of the F component (2b) and dimerization; oligomerization (3) and insertion (4) occur. Modified and adapted from [168, 175].

A common feature of PVL, γ-hemolysin and LukED is that they all target chemokine and complement receptors, which might result from the high sequence similarity of the toxins with each other (60-80%) [176]. In contrast, the LukAB sequence matches the other toxins by 30-40% and instead, it targets an integrin subunit (**Table 4**) [176-178].

PVL is present in only 5% of all strains and those PVL-positive strains are epidemiologically associated with severe infections such as necrotizing pneumonia [179, 180]. However, there are also contradictory studies and opinions regarding the role of PVL in infections. Some studies suggest that PVL is not the major factor contributing to disease, while others argue that the presence of PVL can even be beneficial for the outcome of the infection [176]. However, the fact that PVL only elicits a cytotoxic effect in rabbit and human cells makes results acquired from murine and primate animal models questionable [181]. LukS-PV targets C5aR and C5L2 [182] and can contribute to acute inflammation by inducing the release of IL-8, histamine and $LTB_4$ [168]. At sub-lytic concentrations it has been shown that PVL can alter gene expression in neutrophils, which leads to enhanced killing of *S. aureus* (**Table 4**) [183].

The two forms of γ-hemolysin (HlgAB and HlgCB) have different S subunits (HlgA or HlgC) but share the same F subunit (HlgB) [184] and are found in 99% of all strains

[185]. γ-hemolysin also targets red blood cells and it has been shown that the genes hlgA, hlgB and hlgC are up-regulated in the blood stream during infection and this contributes to *S. aureus* survival [186]. HlgAB targets the chemokine receptors CXCR1 and 2 and CCR2, while HlgCB targets the complement receptors C5aR and C5L2 (**Table 4**) [187].

**Table 4. Characteristics of *S. aureus* α-toxin and leucocidins.** Modified and adapted from [168].

| | toxin | Component | | cell type | receptor | strain distribution | species specificity | sublytic activity |
|---|---|---|---|---|---|---|---|---|
| | | S | F | | | | | |
| | **α-toxin** | | | RBC, epithelial cells | ADAM10 | 99% | Yes, rabbit > human | proinflammatory |
| **Bi-component leucocidins** | **PVL** | LukS-PV | LukF-PV | **neutrophils**, monocytes, macrophages | C5aR, C5L2R | 2-3% | Yes, rabbit and human only | neutrophil priming, inflammasome activation, induction of apoptosis |
| | **γ-hemolysin (HlgAB/CB)** | HlgA | HlgB | monocytes, macrophages, **neutrophils**, T-cells, RBC | CXCR1, CXCR2, CCR2 | 99% | unclear | unknown |
| | | HlgC | HlgB | | C5aR, C5L2R | 99% | unclear | $Ca^{2+}$ release |
| | **LukED** | LukE | LukD | macrophages, **neutrophils**, DCs, NK cells, T cells, RBC | CCR5, CXCR1, CXCR2 | 70% | No, toxic across species | unclear |
| | **LukAB** | LukA | LukB | **neutrophils**, monocytes, macrophages, DCs | CD11b | unknown | Yes, human and rabbit | unclear |

DC = dendritic cell, NK cell = natural killer cell, RBC = red blood cell

70% of *S. aureus* strains express LukED, which has no species specificity and targets a broad range of different cell types [168, 188]. It lyses macrophages, DCs and T cells by interacting with the receptor CCR5, while in contrast, in neutrophils, monocytes and NK cells the receptors CXCR1 and 2 are used to build a pore (**Table 4**) [189, 190].

The strain distribution of LukAB is currently not known, which might be due to its most recent identification [177, 191]. It can be either secreted or attached to the bacterial cell surface [177]. In contrast to the other leucocidins, LukAB targets an integrin – CD11b – which is part of the Mac1 complex on neutrophils, macrophages and monocytes [178]. Furthermore, it has been shown that LukAB can promote NET formation (**Table 4**) [192].

*S. aureus* leucocidins are powerful tools utilized by the bacteria to prevent killing by phagocytes and they contribute to the outcome of the infection by induction of inflammation and tissue damage [168]. This makes it essential and necessary to gain insight in every single leucocidin interaction to find possible targets for new treatments.

## 1.4 IMMUNE EVASION STRATEGIES

*S. pyogenes* and *S. aureus* are human pathogens that share many characteristics. They are both part of the human flora - *S. pyogenes* colonizes the mouth and respiratory tract in 2-5% of the population, whereas *S. aureus* commonly inhabits the skin and anterior nares (up to 30%). However, they are also capable of causing invasive diseases, such as septic shock. Both pathogens have developed many immune evasion mechanisms to avoid or prevent an immune response [193].

For instance, both pathogens express surface virulence factors, so called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that can recognize and bind extracellular matrix molecules. One of these molecules is fibronectin, which is bound by the streptococcal M protein [194] among others, and by the staphylococcal FnBPA and FnBPB [195]. This binding helps both pathogens to attach to the host (cells or tissue) and to hide from the immune system, when bound to the cell surface.

Another evasion strategy is the avoidance of opsonization by the complement or by antibodies. Clumping factor A [196] from *S. aureus* and M proteins [197] from *S. pyogenes* bind fibrinogen which prevents the binding of the complement opsonization factor C3b. Furthermore, proteases such as the cysteine protease SpeB (*S. pyogenes*) and the serine protease V8 (*S. aureus*) are capable of cleaving C5a and IgG, thus protecting the bacterium [198-201].

An additional shared feature is the ability to inactivate host effector molecules like the antimicrobial peptide LL-37, as seen with SpeB [202] or aureolysin, which is expressed by *S. aureus* [203]. Moreover, the expression of DNases provides an escape mechanism from NETs [27, 138, 204].

Cytolytic toxins such as BCTs and phenol-soluble modulins (*S. aureus),* or the streptococcal hemolysin SLO, can damage host cells such as neutrophils, which resulting in cell lysis. This is a very important mechanism to prevent phagocytic killing [193].

# AIMS

This project builds on the hypothesis that neutrophil activation might not only represent an important bacterial clearance mechanism, but also a central event in the pathogenesis of severe invasive *S. pyogenes* and *S. aureus* manifestations. To this end, the project focused on dissecting the interactions between neutrophils and these pathogens, and aimed to identify bacterial factors involved in neutrophil activation, the resulting neutrophil responses and functional consequences of these bacteria-neutrophil interactions (**Figure 8**).

**Specific aims include:**

- Determine the neutrophil responses to defined *S. pyogenes* clinical strains and identify streptococcal factors involved in neutrophil activation (**paper I**).

- Explore the ability that LL-37, the neutrophil derived effector molecule, has on influencing *streptococcal* virulence (**paper II**).

- Investigate the neutrophil response to defined *Staphylococcus aureus* leukotoxins in relation to their cytotoxic activity (**paper III**).

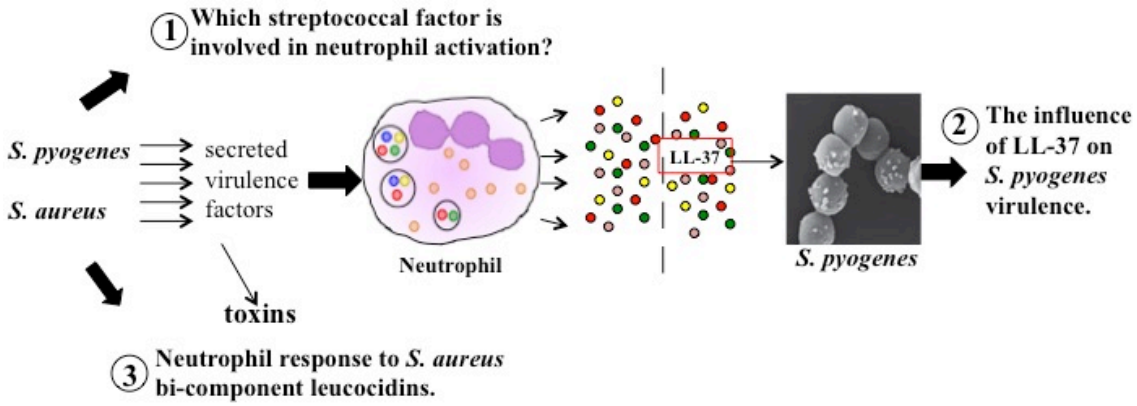



**Figure 8: Schematic presentation of the aims of this PhD project.**

# RESEARCH APPROACH

The experimental techniques used in this thesis are described in detail in the respective papers and manuscript.

**Bacterial work:**

All strains that were used in **paper I-III** are clinical isolates handled with minimum passaging. The use of clinical isolates that have been minimally passaged has the advantage that they are likely to retain their virulent properties. In contrast, laboratory strains have adapted over the years to rich media and a lack of challenges from the host making them less virulent. However, it should be recognized that the *in vitro* culture of the clinical isolates in the rich THY medium for *S. pyogenes* and CCY for *S. aureus* may alter the virulence properties, when compared to the growth in the patients. A challenge posed with *in vitro* experiments is the fact that different culture media and different growth phases will alter the virulence profile of the bacteria. In our studies, we mostly use stationary phase bacteria (typically 18h cultures) grown in rich media, supporting a high exotoxin production (**Figure 9 and 11**). This was chosen as it somewhat resembles the *in vivo* situation, as studies in patients have shown a high bacterial load and greatly elevated expression of exotoxins plus other virulence factors, such as M protein, at the site of infection [119, 149, 205].

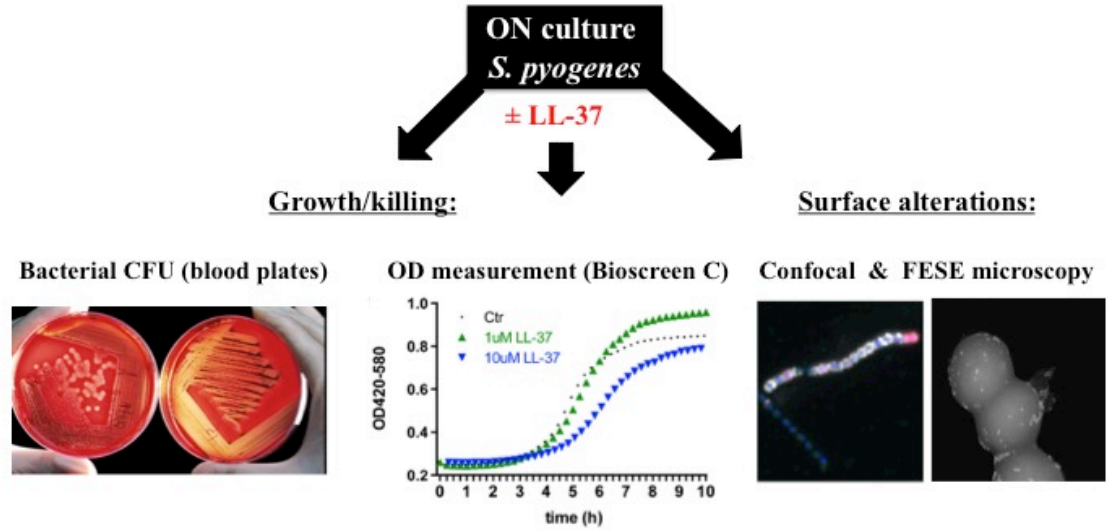



**Figure 9. Experimental approach to study the effect of LL-37 on bacterial growth/killing and on surface alterations.** Streptococcal over-night (ON) cultures (18h cultures) were used for all experiments with incubation of ± LL-37. The growth was analyzed by CFU counts or OD measurement. Morphological surface alterations were studied by confocal and FESE microscopy.

To monitor bacterial growth over time we used the **Bioscreen C assay**, which is a computer-based approach that measures optical density (OD) of bacterial cultures (**Figure 9**). Parameters like temperature, shaking and the time intervals between measurements can be precisely adjusted. This method for measuring the turbidity of cells is ideal for large screening experiments with many parameters and can be used to get a general idea about the growth behavior of different bacterial strains and/or conditions (e.g. +/- LL-37). However, it cannot be directly translated into bacterial CFUs (colony forming units) (**Figure 9**). As shown in **paper II**, the increase in turbidity was linked to the production/release of extracellular-like structures and not to increased bacterial numbers. The experimental approach that we used for investigating the properties of these vesicle-like structures are shown in **Figure 10**.

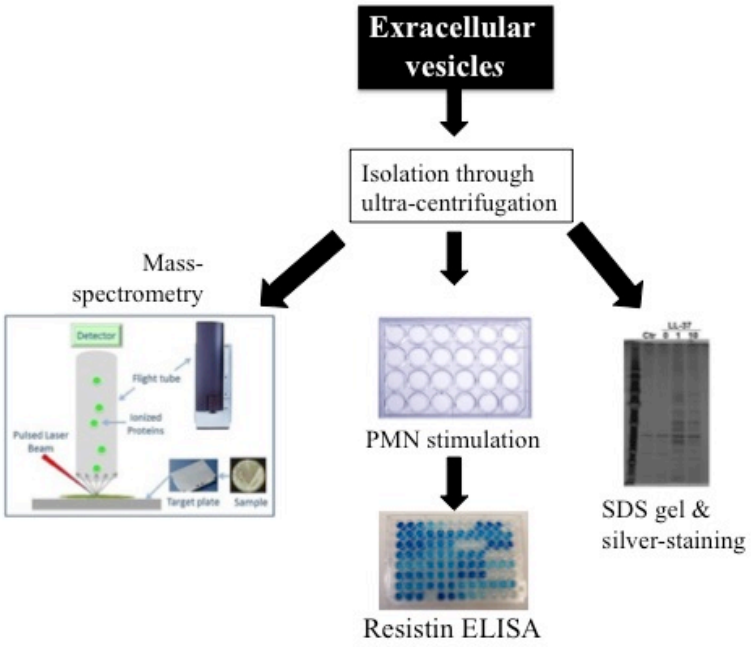



**Figure 10. Experimental approach to study the properties of extracellular vesicle-like structures.** After incubation with LL-37, the bacterial cultures were ultra-centrifuged. The "vesicle-enriched pellet" was used to analyze the protein contents with mass-spectrometry. For visualization and comparison of the protein content, samples were run on a SDS gel and silver stained. The stimulatory effect of vesicles was examined by stimulation of primary neutrophils (PMNs) and neutrophil supernatants analyzed by the amount of resistin release (ELISA).

**Confocal microscopy** is a widely used technique to investigate the interactions between LL-37 and bacteria and gives a good overview of the binding pattern. Therefore we used this technique to compare our findings with other studies, i.e. reports on interaction of LL-37 with the ExPortal [206-208]. However, the resolution was not high enough to visualize interactions and membrane alterations. To investigate where and how LL-37 interacts with the bacterial surface, we utilized the knowledge and expertise of our collaborators in

Germany. By employing **electron microscopy** with much higher magnification and resolution, we were able to look at single peptide-bacteria interactions (field emission scanning electron microscopy (**FESEM**)) as well as morphology changes of the membrane. Transmission electron microscopy (**TEM**) and negative staining of the bacteria induces the contrast of the sample and reveals morphological changes within the cell (**Figure 9**).

**Neutrophil work:**

We used **primary human neutrophils** isolated from whole blood and collected from volunteers for all experiments. Neutrophils are short-lived cells that undergo apoptosis if not used shortly after isolation. Therefore the experiments were planned well in advance. In addition, the time of the isolation plays a crucial role as the immune system is under circadian control [209]; in the beginning of the active phase (after resting) the immune system has a much stronger reaction compared to later in the day [209, 210]. Therefore, we always tried to isolate and stimulate them around the same time. Another drawback with primary cells is the donor variations, which is apparent with the numbers of cells isolated and the neutrophil response profiles to different stimuli. In addition, working with primary neutrophils requires careful handling, as these cells are easily activated during the isolation process. However, to date, there is no suitable human cell line available to study the neutrophil degranulation response. An alternative option could have been the use of the human leukemia cell line HL-60 [211], which can be differentiated into neutrophil-like cells [212]. However, these differentiated cells only contain azurophilic granules and lack the other types of granules and vesicles [213].

In order to analyze the neutrophil response by mass spectrometry, we adjusted the experimental conditions to minimize any disturbance caused by the mass spectrometer. Therefore, we used HBSS (hank`s balanced salt solution) without FCS (fetal bovine serum) or plasma, instead of RPMI.

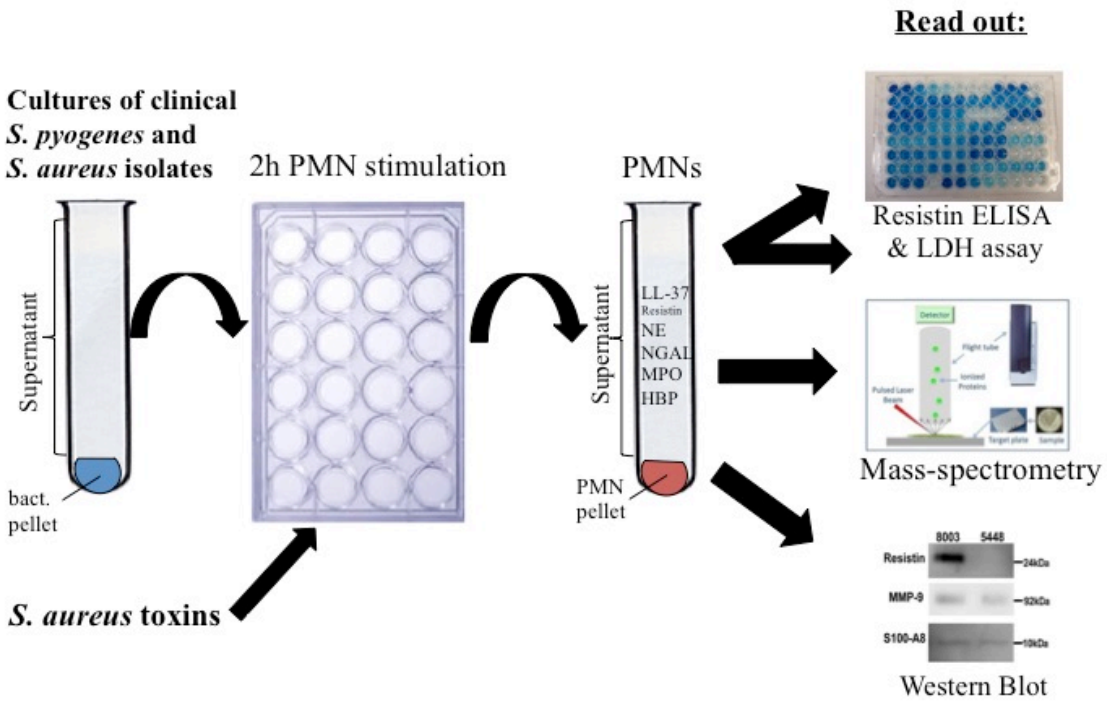



**Figure 11. Experimental approach to study the effect of bacteria towards neutrophils.** Cell-free supernatants from over-night cultures of either *S. pyogenes* and *S. aureus* and/or *S. aureus* toxins were used to stimulate neutrophils (PMNs). The effect on neutrophil activation/degranulation and cytotoxicity was analyzed by measuring resistin and LDH, respectively. The whole neutrophil secretome was evaluated by mass-spectrometry and the presence of selected proteins was confirmed via western blot.

After stimulation with different bacterial components (cell-free supernatants, recombinant toxins or extracellular-vesicles) the neutrophil response was determined by measuring lactate dehydrogenase (**LDH**) as a measure of cytotoxic effects, or the amount of released **resistin (ELISA)** (**Figure 11**). We used resistin as a degranulation marker, as this protein is stored in azurophilic granules and previous work in our group identified that this factor is associated with the severity of sepsis and septic shock [43, 78]. Triton-X was used to determine the total amount of resistin and the cytotoxic response.

To fully characterize the neutrophil secretome profile, proteomic analyses were conducted at the core facility at KI for **Mass-spectrometry** analysis (**Figure 11**). Label-free quantitative mass spectrometry proteomics was used, which revealed information about proteins present in neutrophil supernatants (**paper I and III**). Similarly, mass spectrometry was utilized to identify factors present in bacterial supernatants (**paper I and II**) (**Figure 10**). In addition, **Western Blot** analysis further confirmed the presence of selected proteins found by mass spectrometry (**Figure 11**).

# RESULTS AND DISCUSSION

## 1.5 *STREPTOCOCCUS PYOGENES* VERSUS NEUTROPHILS (PAPER I AND PAPER II)

In **paper I** and **paper II** we focused on *S. pyogenes* and neutrophils. In **paper I** we were interested in the neutrophil response to different streptococcal strains and on identification of novel bacterial factors involved in neutrophil degranulation, whereas in **paper II**, we studied the impact of LL-37 on streptococcus virulence.

### 1.5.1 Streptococcal PGK triggers neutrophil degranulation (paper I)

In this study we investigated the following questions:

a) Are there differences in neutrophil responses towards different group A streptococcal secreted factors? And if yes,
b) Can we identify the key bacterial factors involved in neutrophil degranulation?

#### 1.5.1.1 Neutrophil activation is linked to the absence of SpeB

In this study, we demonstrated that the neutrophil response to different streptococcal supernatants varies depending on the strain tested. Initially we used M1 and M3 strains, as these M types are associated with severe streptococcal infections [214]. The M3 strain 8003 induced a significantly stronger neutrophil response compared to the M1 strain 5448. We could demonstrate that apart from the neutrophil degranulation marker resistin, the whole neutrophil secretome was affected by 8003, as the stimulated neutrophil supernatants contained a broader repertoire of different granule proteins than found in the 5448-triggered neutrophil secretome (**Figure 1, paper I**).

However, this was not linked to the M type, but rather associated with the presence or absence of SpeB in bacterial culture supernatants. A parallel study in our group showed that 8003 lacks the cysteine protease SpeB [215]. This protease is an important virulence factor as it proteolytically degrades numerous host factors but of greater importance in this study, it also degrades endogenous virulence factors [216]. The significance of SpeB absence in 8003, and thereby an increased neutrophil response, was confirmed by the

inclusion of several other streptococcal strains with various M types. The SpeB-negative strains induced a significantly stronger neutrophil response in comparison to SpeB-positive strains (**Figure 2, paper I**). This led us to the hypothesis that the stronger neutrophil response must be linked to a streptococcal factor that is susceptible to SpeB degradation. To test this, SpeB was inhibited by the addition of the E64 protease inhibitor during bacterial growth, and the following neutrophil stimulation showed that the treated SpeB-positive strains induced a much stronger neutrophil response **(Figure 3 B, paper I)**. In addition, we included a *spe*B-deletion mutant, which showed a similar phenotype to the other SpeB-negative strains, namely a stronger neutrophil response in comparison to the wild-type strain (**Figure 3 D, paper I**). These results confirmed our hypothesis that the presence or absence of SpeB determines the effect of the bacteria on neutrophils, and it is also likely that other bacterial virulence factors are involved, if not degraded, by SpeB.

Other studies have shown that there is an association between SpeB-negative strains and increased virulence. Most of these SpeB negative strains arose through mutations in the CovR/S (control of virulence regulatory sensor kinase) system. This two-component system regulates about 15% of the streptococcal genes [217] and mutations are associated with hypervirulent phenotypes [218]. Virulence factors such as SLO, hyaluronic acid, DNase Sda1, M protein, SIC, SmeZ, NAD-glycohydrolase, C5a peptidase and the adhesion collagen-like surface protein are shown to be up-regulated [137, 219]. The DNase Sda1 [137] as well as *hasA* (capsule synthetase) and the *emm* (M protein*)* genes appear to be critical for the selection of SpeB negative mutants in mice [220]. In addition, neutrophils exert pressure on the selection of CovRS mutations in vivo, as seen in neutrophil-depleted mice the number of strains with mutations in *covRS* were decreased [221]. Furthermore, the SpeB expression was higher in strains isolated from non-severe cases in comparison to isolates from severe cases [222].

However, other reports exist that implicate SpeB in disease pathogenesis, such as the recent study by Olsen *et al*., where they looked at a large collection of clinical *S. pyogenes* strains and found the majority of clinical isolates express SpeB [223]. In addition, our group has shown that soft tissue biopsies from patients with necrotizing soft tissue infections contain a mixture of SpeB-positive and -negative strains which were directly isolated from patients diagnosed with necrotizing skin and soft tissue infections [215]. SpeB has many important biological functions and it seems likely that the role differs during distinct stages of the infections. The presence of SpeB might be beneficial in some stages during infections, e.g. after entry through the skin, when nutrition is needed,

whereas in the later stages of infections, SpeB-negative strains might have an advantage, e.g. by not degrading Sda1, and thereby escaping NETs. At present, the role for SpeB in streptococcal infections remains elusive, controversial, and needs further investigation.

### 1.5.1.2 PGK is a novel neutrophil activator

To investigate which of the secreted bacterial factors are involved in the aforementioned neutrophil activation, we used supernatant from the SpeB-negative strain 8003. Heat-inactivation of the supernatant resulted in a loss of the stimulatory capacity towards neutrophils. This indicated that the stimulatory factor is most likely of protein nature (**Figure 4A, paper I**). To further identify particular factors involved in neutrophil degranulation, we fractionated the bacterial supernatant based on protein size. The fraction with the highest stimulatory capacity towards neutrophils (F2) and also a high protein content (**Figure 4 B and C, paper I**), was used for mass spectrometry analysis. Amongst the identified proteins, there was one protein of particular interest – the phosphoglycerate kinase (PGK).

Only few reports have investigated the role and function of this protein. PGK is a glycolytic protein and converts 1,3-bisphosphoglycerate to 3-phosphoglycerate by producing ATP. In addition, there are reports which identified PGK as one of the 5 anchorless adhesion molecules present on streptococcal surfaces [124]. Streptococcal surface proteins can be associated with the bacterial cell wall in four different ways; with peptidoglycan by a LPxTz motif at their C-terminus, through their N-terminal region, via non-covalent interactions, or lastly, by a so far unknown mechanism [224]. There are reports that the surface associated Group B *Streptococcus* and *S. pneumoniae* PGKs interact with plasminogen and actin, providing a role as adhesion proteins [225-227]. In addition, the pneumococcal PGK inhibits the classical and alternative complement pathway by interacting with the membrane attack complex [228]. These other functions, which are likely important for virulence, suggest that PGK belongs to the group of moonlighting proteins [229]. So far, no additional functions besides the role in glycolysis have been reported in *S. pyogenes*. Therefore, we wanted to test the hypothesis that PGK is a potential factor involved in neutrophil activation.

First we tried to generate a knock-out mutant despite the fact the survival rate prediction for a PGK-mutant was only 40%. The efforts to generate a mutant failed, which suggested

that this mutation might be lethal as PGK is involved in essential bacterial processes, namely the generation of energy in glycolysis. Nevertheless we were able to construct a recombinant PGK, which we purified and used in further experiments to investigate (i) SpeB-susceptibility and (ii) the stimulatory capacity of this protein towards neutrophils. By utilizing SpeB-positive and –negative supernatants from 5448 and 5448AP strains, we showed a time-dependent degradation of PGK by the SpeB-positive supernatants (**Figure 6 A, paper I**). The exposure of primary neutrophils to different concentrations of PGK alone, or in combination with 5448 supernatant resulted in a dose dependent degranulation response. However, the highest response was noted in the samples where PGK was added to 5448 supernatants with the SpeB inhibitor E64 (**Figure 6 C, paper I**). This suggested that there are additional co-stimulatory factors leading to increased neutrophil activation.

In conclusion, in this study (**paper I**), we identified a novel neutrophil stimulatory factor, namely PGK, which was found in SpeB-negative streptococcal supernatants. The fact that we only found PGK in SpeB-negative strains indicates the broad effector range of SpeB`s degradation potential. The occurrence of SpeB-negative strains and their association with severe infections suggests that PGK might contribute to the outcome of severe streptococcal diseases (**Figure 12**).

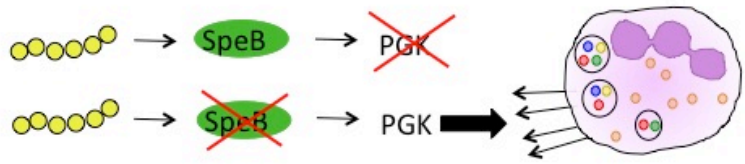



**Figure 12. Schematic presentation of the results from paper I.**

### 1.5.2 LL-37 triggers bacterial vesicle-like structures (paper II)

Although it has been shown that LL-37 has a great antimicrobial activity against different bacteria, *S. pyogenes* has developed different counter strategies to evade the antimicrobial effect (**Table 5**) [230]. As LL-37 is attracted by negatively charged surfaces, *S. pyogenes* repels the antimicrobial action by three mechanisms that have been identified so far: a) by expressing an enzyme that increases the charge of the surface through D-alanylation [231], b) by protecting its surface membrane with a hyaluronic acid capsule [220] and c) by integrating a specific carbohydrate – glucoroic-B1,3-N-acetylglucosamine – in its cell wall, which blocks the LL-37 interaction [232].

SIC (serum inhibitor of complement) and M1 protein, are able to interact with LL-37 directly. The secreted factor SIC [233, 234] and the M1 protein [235], which is attached to the surface, bind directly to LL-37 and thereby prevent the antimicrobial action of LL-37 on the membrane.

**Table 5. *S. pyogenes* counter strategies to evade LL-37 killing.** Modified and adapted from [230].

| Category | Specific mechanism | References |
|---|---|---|
| Repel: | | |
| DltABC | Lipotechoic acid D-alanylation | [231] |
| HasABC | Hyaluronic acid (capsule) | [220] |
| GacA-L | Glucoronic-B-1,3-N-acetylglucosamine | [232] |
| Intercept: | | |
| M1 protein | Competitive binding | [235, 236] |
| SIC | Competitive binding | [233, 234] |
| Destroy: | | |
| SpeB | Direct proteolysis | [134] |
| GRAB | Protease recruitment/redirection | [133] |
| Ska | Activation of proteolysis by plasmin | [237] |

As mentioned earlier, the secreted cysteine protease SpeB is able to cleave host factors, including LL-37 [134]. Even when SpeB is trapped in the binding complex of the surface bound G-related α2-macroglobulin-binding protein (GRAB) with the circulating α2-macroglobulin, a host protease inhibitor, SpeB is still able to cleave and degrade smaller substances which are small enough to enter this " SpeB cage" [133]. Thus, *S. pyogenes* creates an LL-37 inhibitory zone on its surface. A further indirect method of destroying LL-37 is facilitated by Ska, which recruits plasminogen and cleaves it to its active form

plasmin. Plasmin in return then cleaves LL-37 and protects *S. pyogenes* against its antimicrobial activity [237].

### 1.5.2.1 LL-37 effect on bacterial growth

In this study we investigated the effect of sub-lytic concentrations of LL-37 on the growth behavior of *S. pyogenes* and its impact on streptococcal virulence properties. First, strain 5448 was exposed to different concentration of LL-37 (1-10 μM) and the growth behavior of the culture was monitored by assessing turbidity via Bioscreen C. The analysis showed that there is a significant increase in OD/turbidity when the bacteria were incubated with 1 μM LL-37 (**Figure 1a and d, paper II**). However, this was not reflected in the CFU numbers of the same bacterial cultures (**Figure 1e, paper II**).

To investigate if the observed effect was limited to strain 5448, or if it is more general in nature, we included other clinical isolates of different M-types in the analysis. Except for turbidity, we also analyzed the minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC). The data showed that the response to LL-37 varies between the strains. There was even inter-strain variation within the same M-type noted. In addition, there was no correlation noted between increased turbidity and MIC and MBC.

### 1.5.2.2 LL-37 induces bacterial surface alteration

Since the increase in turbidity seen in some of the strains was not linked to bacterial growth, we employed confocal microscopy to visualize the interactions between the peptide and streptococci. After 30 minutes of exposure, we noticed that LL-37 was bound to a limited number of bacterial chains (**Figure 3, paper II**). This might be explained by the fact that LL-37 forms oligomers [238]. It is likely that at low concentrations, these LL-37-oligomers reach only a few bacterial chains. In addition, it appeared that LL-37 targeted the bacterial plane of division (**Figure 3, paper II**). This was in line with the observation by Kristian *et al.*, where LL-37 preferentially targeted *E. coli* that were in the process of dividing [239].

However, the usage of electron microscopy and immune-gold labeling of the LL-37 antibody revealed that LL-37 did not bind exclusively to the plane of the division, but it

was also found in several other areas of the bacteria (**Figure 5 c-f, paper II**). Of special interest was the observation of vesicle-like structures protruding from the bacteria membrane in bacteria exposed to either 1 μM or 10 μM LL-37 (**Figure 5 c, d; arrows, paper II**). This was unexpected, as vesicle formation has been a well-known feature of Gram-negative bacteria for more than 50 years, but not much is known about the production of vesicles in gram-positive bacteria.

In Gram-negative bacteria, outer membrane vesicles (OMVs) are actively formed by pinching off parts of the outer membrane and they have been shown to have a role in bacterial pathogenesis and immune regulation [240]. OMVs contain parts of the outer membrane, LPS, periplasmic and membrane-bound proteins, enzymes, toxins, DNA, RNA and peptidoglycan. The controlled release of OMVs has an impact on bacterial virulence, and in addition it has been shown that vesicle release can be related to stress, and it also helps the bacteria to communicate with one another plus modulates the host immune responses [240-243].

Only recently, in the last couple of years, extracellular membrane-derived vesicles (MVs) have been described in Gram-positive bacteria, such as *Staphylococcus aureus* [244], *Bacillus anthracis* [245], and *Streptococcus pneumonia* [246].

### 1.5.2.3 Vesicles formation and impact on neutrophils

The use of electron microscopy and negative staining of the bacteria confirmed the initial finding that LL-37 induces the release of vesicle-like structure on the cell surface of streptococci (**Figure 4 a-f, paper II**). Bacteria treated with high concentrations of LL-37 (10 μM) showed larger aggregates on their surface, which were connected with the cytoplasm. This was also confirmed by field emission scanning electron microscopy (FESEM) analysis (**Figure 4 i, paper II**). About the same time as we found that sub-lytic concentration of LL-37 can induce vesicle release (**paper II**), another study showed that sub-lethal concentrations of penicillin have a similar effect on *S. pyogenes* [247]. This led us to investigate the content of the vesicle structures. We identified several proteins such as enolase and GAPDH. These proteins belong to the same group as anchorless adhesion molecules like PGK. In addition, within the vesicles we also detected M protein, streptokinase A, and streptolysin, amongst others (**Table 2, paper II**). Exposure of primary human neutrophils to the vesicle-like structures revealed the immunostimulatory

capacity of the vesicles, as seen through neutrophil activation and degranulation (**Figure 7 c and d, paper II**). This was an important finding as invasive streptococcal infections are associated with hyper-inflammatory conditions, such as high numbers of infiltrated immune cells, high bacterial load and the presence of LL-37 [248].

### 1.5.2.4 The role of *CovS* in vesicle formation

Furthermore, we explored the mechanism behind the LL-37 induced vesicle formation. A report has shown that 10-residues found in LL-37 (RI-10), which lack antimicrobial activity, are required for direct interaction with CovS [249]. Our experiments revealed that RI10 is also sufficient for inducing vesicle formation, implicating the involvement of CovRS in vesicle formation (**Figure 6 a, right image, paper II**). However, by utilizing the natural *cov*S mutant 5448AP, which has defective signaling, as well as 581Δ*cov*S, we showed that these mutants produced vesicles, although they were released in larger aggregates in response to LL-37, similar to those seen in the response of 5448 to 10 μM LL-37. This indicated a greater antimicrobial permeation effect, which was in line with their lower MIC and MBCs when compared to their respective wild-type strain (**Table 1, paper II).** Resch *et al*. [250] recently investigated the mechanisms behind membrane-derived vesicle formation in *S. pyogenes* and specifically the impact of CovRS on vesicle formation and content. They used different *covS* mutants; one was a *covS*-defective strain NS88.2 (emm type 98.1) and the other had a large deletion in *covS (*ISS3348, M1 Type*),* as well as 5448AP (also used in **paper II**). All *covS* mutants showed a significant increase in vesicle production in comparison to their respective wild-type strain or "repaired" strain, by quantification per CFU; this suggested that CovRS had a negative impact on vesicle formation [250]. This is similar to our finding, as we saw larger aggregates in the *cov*S mutant consistent with a greater antimicrobial effect induced by LL-37, rather than increased vesicle formation. Nevertheless, we cannot exclude the involvement of CovS and whether or not the effect of CovS is of direct or an indirect nature remains unclear. In addition Resch *et al.* investigated the involvement of SpeB and the capsule, as *Cov*S mutations are associated with the loss of SpeB expression and up-regulation of the capsule. However, both had no effect on vesicle formation [250].

In conclusion, this study shows that sub-lytic concentrations of LL-37 can induce vesicle formation in *S. pyogenes,* which may contribute to pathogenesis as the protein content induced an inflammatory response in neutrophils (**Figure 13**). We showed that the 10

residues which interact with CovRS (RI-10) [249] and lack antimicrobial activity, were sufficient to induce vesicle formation. However, a deletion mutant which lacked CovS signaling, and mutant with a covS mutation, showed the same phenotype, namely the release of larger aggregates, similar to the effect seen with higher LL-37 concentrations and wild-type bacteria. This suggested that CovRS signaling seems to impact the effect of LL-37 on vesicle formation but our data suggests that other mechanisms might be involved as well.

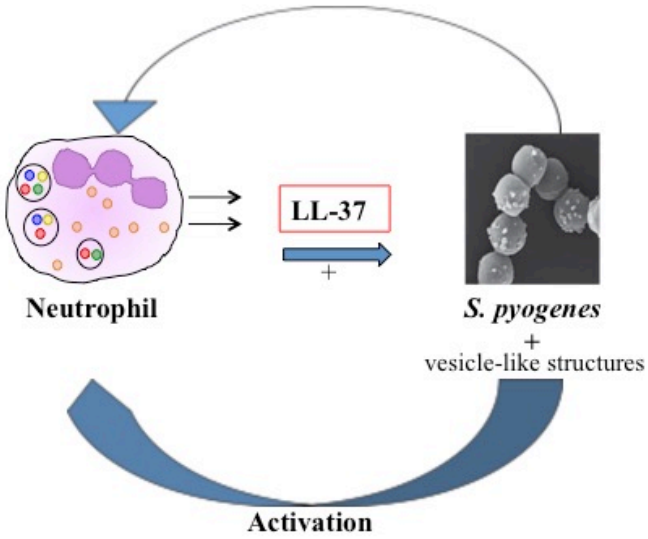



**Figure 13. Schematic presentation of the results from paper II.** Thin arrow indicates a weak, thick arrow a strong neutrophil response.

## 1.6 *STAPHYLOCOCCUS AUREUS* VERSUS NEUTROPHILS (PAPER III)

### 1.6.1 Neutrophil responses triggered by leukotoxins (paper III)

Similar to *S. pyogenes*, *S. aureus* have evolved immune evasion strategies to avoid the immune response, including phagocytic killing by neutrophils. Therefore, it expresses a variety of cell surface and secreted virulence factors that help the bacteria to colonize humans or cause a range of infections from uncomplicated skin ones to the very severe, such as necrotizing fasciitis or pneumonia. In addition, the bi-component leukotoxins (BCLs) are important virulence factors, as they are able to form pores in neutrophils, amongst others, which can induce the uncontrolled release of granule proteins. However, besides their cytotoxic effect, activation and degranulation of neutrophils has not been in investigated in detail.

#### 1.6.1.1 Neutrophil degranulation is independent of cytotoxicity

In this study we wanted to investigate the neutrophil response to defined BCLs in *S. aureus* supernatants with a focus on activation and cytotoxicity towards neutrophils. First, we looked at the clinical isolate LUG2012 (USA300 strain) and its isogenic mutants PVL and α-toxin deficient mutants. We saw a significant decrease in cytotoxicity towards neutrophils with the PVL mutant, while resistin release was not affected (**Figure 1 A and B, paper III**). The α-toxin was used as a negative control as it has been shown to target other cell compartments, such as epithelial and red blood cells, rather than neutrophils [173, 251]. As expected the α -toxin knock-out had no significant effect on neutrophil lysis or degranulation, compared to the wild-type strain (**Figure 1 A and B, paper III**).

The finding that a strong resistin response was even seen after stimulation with the PVL-mutant (despite its loss of cytotoxic activity), was interesting and we wanted to explore whether this was a general feature for BCLs. For this purpose we utilized an additional strain, SF8300 (also a USA300 strain), and its isogenic mutant strains deficient in PVL, LukED, and LukGH. The ΔLukGH mutant showed a similar cytotoxicity and resistin profile compared with the wild-type strain (**Figure 1 C and D, paper III)**. Both mutants - ΔPVL and ΔLukED – induced less cytotoxicity. This loss of cytotoxicity was stronger for

ΔPVL in comparison to ΔLukED. However, the reduction in cytotoxicity was not reflected in changes in the levels of resistin as these were similar in all strains (**Figure 1 C and D, paper III**). Thus, the data shows clear differences between the toxins, with PVL even inducing resistin release at sub-lytic concentrations, whereas LukED requires lytic concentrations for resistin release.

To exclude the role of additional secreted factors involved in the noted effect seen with the PVL and LukED mutants, we used recombinant toxins. Both toxins induced a dose-dependent cytotoxic effect towards neutrophils (**Figure 2 A, paper III**). In agreement with the results from the bacterial mutants, with regards to resistin release, LukED induced a dose-dependent release, whereas PVL induced an equally high resistin response in all concentrations tested (**Figure 2 B, paper III**). Consistent with this finding, we found a positive correlation between cytotoxicity and resistin for LukED ($p<0.0001$) but not for PVL ($p<0.014$) (**Figure 2 C, paper III**).

The S component of LukED (LukE) and PVL (LukS-PV) share 69% similarity while the F component LukD and LukF-PV share 82% similarity [176]. Both toxins target chemokine receptors. While PVL binds to neutrophils, monocytes and macrophages by targeting the receptors C5a and C5L2, LukED interacts with CCR5 on neutrophils, RBCs, monocytes, macrophages, DCs, NK cells and T cells (**Table 4,** page 23) [168]. Major differences between these two BCLs include the strain distribution and species specificity. While PVL is only found in approximately 5% of the clinical isolates and exerts its pore-forming effect in only rabbit and human cells, LukED is found in the majority of strains (70%) and is toxic across species [168]. In addition, the cytotoxic dose of PVL in human cells ranks between 80-100 ng/ml while LukED is only cytotoxic at higher doses, around 2-5 μg/ml [252]. Despite their sequence similarity and the fact they target the same kind of receptor, their effect on neutrophils seems to differ in relation to degranulation, while the cytotoxic effects remain similar with a clear dose response effect, albeit at different concentrations.

### 1.6.1.2 Complex neutrophil responses towards LukED and PVL

To further characterize the noted difference in neutrophil response and particularly degranulation/exocytosis, we analyzed, by mass spectrometry, the neutrophil secretome triggered by sub-lytic/lytic concentrations of PVL and LukED. A large number of

significantly differentially expressed proteins were identified following the different stimulations, as compared to unstimulated cells. When comparing the up-regulated proteins with regards to protein networks, the sub-lytic concentration of PVL (40 ng/ml) induced the up-regulation of proteins that are mainly involved in immune responses (**Figure 3 B, paper III)**. Granule proteins such as neutrophil elastase (ELANE), MPO and HDPs (LL-37 and defensins 1 and 3) were expressed to a significantly higher extent when compared to those seen with lytic concentrations of PVL and LukED (**Figure 3 C, paper III**).

In contrast to PVL40, the lytic concentrations of PVL and LukED induced a more heterogeneous response profile, with changes seen in proteins involved in immune responses, the actin cytoskeleton, protein polymerization and other cellular processes (**Figure 3 B, paper III**). Selected proteins, such as the high mobility group protein B1 (HMGB1), showed a higher expression in the lytic toxin concentrations than with PVL40, which is in line with HMGB1 being an alarmin (DAMP) released by necrotic cells (**Figure 3 C, paper III**) [253]. In addition, the two proteins annexin A11 (ANXA11) and S100P were up-regulated under all three toxin conditions when compared to unstimulated cells (**Figure 3 D, paper III**). Both proteins are involved in $Ca^{2+}$ signaling and S100P together with HMGB1 belong to the group of alarmins. They are released in response to necrotic and damaged cells and are associated with several inflammatory diseases (**Figure 3 D, paper III**) [253, 254].

### 1.6.1.3 Potential involvement of JNK in neutrophil lysis

Next, we sought to identify signaling pathways involved in aforementioned effects. The secretome-network analysis indicated the involvement of different proteins in signal transduction, such as the Fc-gamma receptor IIIB (FCGR3B), Myristoylated Alanine-Rich C-Kinase Substrate **(**MARCKS), and 14-3-3 protein beta/alpha (YWHAB) (**Figure 3 D, paper III**). Therefore, we used inhibitors targeting different steps in MAP kinase signaling (i.e. inhibitors for: Src-family kinases, PI3K, MAPK p38, ERK1/2 and JNK). The JNK pathway inhibitor (SP) was almost able to completely abolish the cytotoxic effect of LukED, while resistin release was partially reduced with all inhibitors used. In contrast, no blocking effect in PVL stimulations was noted (**Figure 4 A and B, paper III**). The inhibitory effect of SP on LukED, but not on PVL cytotoxicity is another indication of the noted differences towards neutrophil activation between these two

toxins. The potential involvement of the JNK pathway, is in line with the findings by Yeh *et al.,* where it was shown that this pathway is essential for neutrophil killing of *S. aureus* [255]. However, this particular study used live bacteria, which makes it difficult to draw any conclusions in comparison with our study.

In conclusion, our finding points towards different mechanisms for the two BCLs LukED and PVL upon interaction with neutrophils. While the cytotoxic and degranulation response towards LukED is dose-dependent, PVL induced a similar resistin response, even at sub-lytic concentration and an immune response related secretome profile (**Figure 14**). Other reports have described that sub-lytic concentrations of PVL have different effects on neutrophils, besides pore formation. One study reported, that sub-lytic concentration of PVL could alter neutrophil gene expression, which results in enhanced bacterial killing [183]. Other studies noticed a priming effect [256], induction of apoptosis [257, 258] and the release of pro-inflammatory cytokines [258]. These observations might explain why *S. aureus* infections of PVL-positive isolates are associated with a more severe outcome. However, the role of PVL is also discussed in association with a better outcome for the patients [259-261] and furthermore, there are other studies in which no correlation between PVL and the severity of infection has been found [262-264]. Our results suggest yet another effect of sub-lytic PVL concentrations, namely induction of neutrophil granule exocytosis. Typically, excessive release of these potent granule proteins that have been identified in the neutrophil secretome, are associated with inflammatory responses and direct toxic effects on cells and tissue. However, this remains to be addressed in further studies.

| PMN response | PVL | LukED |
|---|---|---|
| cytotoxicity | dose-dependent | dose-dependent |
| activation | sub-lytic and lytic concentrations | only at lytic concentrations |
| secretome | Sub-lytic conc. induced a broad secretome dominated by immune related proteins | sub-lytic conc. showed no alterations |
| | Lytic conc. induced heterogeneous secretome profiles | |
| signaling | JNK pathway<br>$Ca^{2+}$-dependent signaling<br>FcgR-dependent signaling | |

**Figure 14. Schematic presentation of the results from paper III.**

# CONCLUDING REMARKS AND FUTURE ASPECTS

The studies of my PhD projects focused on neutrophil interplay with two Gram-positive bacteria, *S. pyogenes* (**paper I** and **II**) and *S. aureus* (**paper III**); in particular, in light of a potential contribution of neutrophils to disease pathogenesis. For this purpose we explored the interaction between neutrophils and characterized clinical isolates or purified virulence factors. The project sought to identify bacterial factors involved in neutrophil activation, the resulting neutrophil response profile and functional consequences elicited by neutrophil derived factors (i.e. LL-37).

## ***S. pyogenes* and neutrophils**

### Paper I:

Here we explored the impact of *S. pyogenes* secreted factors on neutrophil activation and degranulation and the studies revealed:

- A marked variation in neutrophil degranulation was noted between different *S. pyogenes* strains.
- SpeB-negative strains induced a significantly stronger neutrophil response as compared to SpeB-positive strains.
- PGK was identified as a novel neutrophil immunostimulatory factor.
- This places *S. pyogenes* PGK among the multifunctional moonlighting proteins that have essential roles in glycolysis but also exerts important virulence functions.

The results showing that PGK and neutrophil activation/degranulation were only seen in SpeB negative strains is of interest in light of reports describing hypervirulent SpeB-negative clones that are associated with severe infections. Our data suggests that the presence of SpeB-negative variants could result in a greater streptococcal-triggered neutrophil activation and degranulation, thereby causing exacerbated tissue damage, inflammatory response and systemic toxicity. The clinical relevance of PGK triggered inflammation in patients, and the link with SpeB-negative variants remains to be proven in future studies. It would also be of interest to investigate if other moonlighting proteins, such as GAPDH, would have similar stimulatory effects on neutrophils or other immune cells.

**Paper II:**

In this study, we investigated how the antimicrobial peptide LL-37 influenced *S. pyogenes* with respect to growth, surface architecture and virulence. The study demonstrated that:

- Sub-inhibitory concentrations of LL-37 induce the release of extracellular vesicle-like structures on the bacterial surface.
- These vesicle-like structures contained several virulence factors with stimulatory activity towards neutrophils.
- CovRS interaction with LL-37 has an impact on vesicle formation, however it seems that other factors might also be involved.

Vesicle formation is a part of a secretion-delivery system in Gram-negative bacteria and has a great impact on bacterial pathogenesis. However, not much is known about vesicles in Gram-positive bacteria, and this phenomenon was only recently discovered in a few species. Our study was the first report of *S. pyogenes* forming extracellular vesicle-like structures in response to LL-37. We could not exclude CovRS involvement in vesicle formation and weather this is an indirect or direct effect of CovRS needs to be explored further.

## ***S. aureus* and neutrophils**

**Paper III:**

We also investigated the effect of the pore-forming *S. aureus* toxins, PVL and LukED, on granule exocytosis in relation to toxin-mediated cytotoxicity, and we showed that:

- LukED has a dose-dependent effect towards neutrophils, both in regards to cytotoxicity and activation.
- In contrast, PVL induced a dose-dependent cytotoxicity, but even sub-lytic concentrations triggered neutrophil degranulation.
- JNK-pathway inhibitors affected neutrophil degranulation, suggesting a possible role in neutrophil response towards BCTs.

Our results demonstrated that PVL and LukED have different effects on neutrophils and that neutrophil degranulation is an independent event and does not require lysis. A possible reason for the noted differences between toxins might be that they are targeting

different receptors and thereby different signaling pathways. Additional studies are necessary to follow up on the potential involvement of the JNK pathway, $Ca^{2+}$ - and FcgR-dependent signaling. Other remaining questions include how the more potent neutrophil activation of PVL influences disease pathogenesis, and do other pore-forming toxins exert similar effects.

This project builds on the hypothesis that neutrophil activation might not only represent an important bacterial clearance mechanism, but also a central event in the pathogenesis of severe invasive *S. pyogenes* and *S. aureus* manifestations. To this end, the project focused on dissecting the interactions between neutrophils and these pathogens, and aimed to identify bacterial factors involved in neutrophil activation, the resulting neutrophil responses and functional consequences of these bacteria-neutrophil interactions.

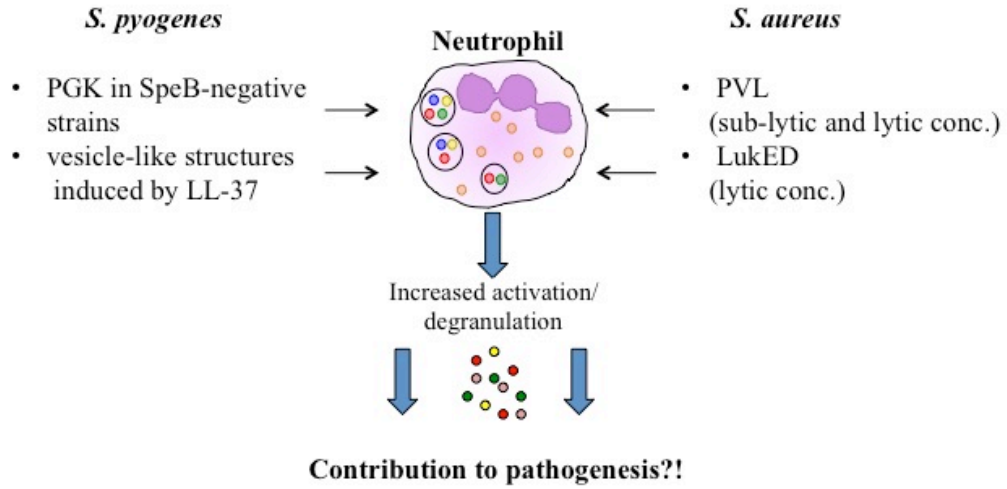



**Figure 15. Schematic summary of results of paper I-III.**

In summary, we identified the streptococcal factor PGK, as well as the pore-forming toxins from *S. aureus* as potent triggers of neutrophil activation and degranulation (**Figure 15**). In addition, we show that the antimicrobial peptide LL-37 induces the release of pro-inflammatory extracellular vesicle-like structures from the surface of *S. pyogenes*. The studies comprised in this thesis contribute to a deeper understanding of the host-pathogen interplay during severe *S. pyogenes* and *S. aureus* infections, in particular with regards to neutrophil activation and a likely contribution to pathogenesis.

# ACKNOWLEDGEMENTS

From viral infections and antigen-presentation to Gram-positive bacteria and neutrophils. From Lund to Stockholm. What started initially with an internship in the immunology section at BMC in Lund has turned into five and a half years in Sweden - and I am about to defend my thesis. Uidiiii!!!:) It wasn`t always easy, with some ups and downs – both scientifically and also privately (Stockholm apartment hunts!). But it makes me proud that I have come to this point now and I would like to thank all these people who have contributed to this:

Firstly I would like to thank my main supervisor **Anna Norrby-Teglund** for giving me the opportunity to complete my PhD at Karolinska Institutet (CIM). You are the most time efficient person I know. I admire your way of scientific thinking, your enthusiasm and how you manage everything. Even in the most stressful times you always had "5 minutes" for me. ;)

**Linda Johansson** (Jervelius), my previous main supervisor and now co-supervisor. Thank you for introducing me to the *Streptococcus pyogenes* world and to LL-37. Your positivity and happy laughter were missed after you left.

**Peter Bergman**, my co-supervisor. Thank you for helping me initially to find a place to stay and for offering your own home as a back-up. You are a really nice person and I have enjoyed discussing science and my projects with you.

**Nikolai Siemens**, for being a good friend, colleague and co-supervisor and for sharing your "Strep" knowledge with me. What would we have done without your straight to the point attitude and you saying things out loud. ;)

**Kajsa Paulsson**, thank you for giving me the opportunity to do an internship in your group. You are such a heart-warming person, great friend and you have been the best mentor I could have imagined during my PhD.

**Anne Halenius**, my diploma supervisor. Without your recommendation to contact Kajsa, I wouldn`t have done my PhD here in Sweden.

**Birgitta Agerberth**, **Gudmundur Gudmundsson** and **Henk Haagsman.** Thank you for the scientific and non-scientific discussions and company during the conference in Portugal 2016.

Many thanks to my collaborators: **Manfred Rohde,** for letting me in to your electron microscopy world and for taking these great images. **Tomas Fiedler**, for sharing *Strep* recombinant proteins with us. **Francois Vandenesch,** for sending us additional *Staph* mutants.

**Josefin Lysell**, thank you for being interested in my LL-37 vesicle finding, discussing it with a more clinical perspective and for your advice.

Many thanks to all the **blood donors,** who volunteered and thanks to the people I was "bothering" to take the blood for me. Without your help I could not have performed all the experiments.

To all my present and past group members: **Marton** alias **Janos**, for introducing me to the lab, to the *Streptococcus* work and for helping me settling down in Stockholm. **Bhavya,** for being such a friendly and warm-hearted person. **Johanna S.,** for being our calming pole in the group - nothing seems to tip you off balance. I admire that and thank you for your help, especially in the end. **Nikolai,** for all the science and non-science discussions, for the hand-on tips in the lab and for being a good friend. **Srikanth**, thank you for the discussions about *Staph* and other scientific questions, for your company and for your "should be fine" problem solving. **Helena, Takeaki** and **Anna L**. for the scientific and non-scientific discussions. Thank you all for the nice group spirit and the fun times we have had in and outside the lab.

Our "Kitchen" office: **Anna R., Ben, David, Emma, Ginny, J-B, Joana, M.C.** and **Puran**. I could not imagine a better office. Sometimes a bit too loud due to the close proximity to the kitchen but we had the best working heating and a never-ending supply of candies. :) Thank you for all the scientific and non-scientific discussions, for sharing calories (candies, ice-cream and sometimes even snaps), vocabulary on our white board and for the great spirit in our office. I will miss that. I've already missed it during the time of writing this thesis.

**Christine, Joana, Nicole** and **Terezia,** for being really good friends and colleagues. Thank you for all the adventures and the fun time we've had and will still have. ;)

Special thanks to **Ginny**, for checking the language in the thesis and for having interesting, early morning discussion; sharing experience about "Ohrschläpperliiies" and co. ;)

Many thanks to past and present CIM members I have met during my time: **Anh Thu**, **Aline, Aline v. A., Alvaro, Carles, Caroline, Dominic, Ebba, Edwin L., Edwin H., Egle, Elza, Erna, Giovanna, Hana, Heinrich, Hongya, Jakob T., Jagadeesh, Jelve, Johanna E., Johanna T., Jovana, Kim, Kimia, Lamberto, Lena B., Lisa, Luca, Magda, Margit, Marianne, Martha, Matthias V., Martin, Michal, Mily, Misty, Moni, Monika, Natalie Si., Natalie Sl., Otto, Pär, Pablo, Renata U., Renata V., Sanna, Salah, Sam, Sayma, Sebastian, Senait, Sofia, Soile, Su, Sush, Tim H., Themis, Tyler, Vikki, Vivien**, all other **PhD students** and **Post Docs** which I might have forgotten and **PI`s** at the department - for making CIM such a nice working space.

Special thanks to **Emma** and **Kim** for providing help in thesis administrative stuff and for sharing your experiences. ;)

**Margit**, for being the best administrator. What would we do without you?! **Lena, Annette, Elisabeth, Hernan** for taking care of the lab and ensuring everything runs smoothly.

I would like to thank the people from BMC in Lund. Especially **Adnan, Aymeric, Camilla, Elna, Linda, Malin and Mo**. You are such great people and it has been fun to work and spend time with you.

Many thanks to my friends in Germany and to all my friends and the people I have met in Lund, Malmö and Stockholm, especially the ones on the dance floor and my climbing buddies (**Aline, Anja, Ben, Joana, Martha**, **M.C. and Su**).

Last but not least I would like to thank my family: Thank you for your unlimited support, for your understanding and for believing in me. Thank you for always being there for me. **Luzy, Micha, Oko, Heri, die Katschas, Urmelchen, Opa Willi** (RIP), **die jungen Katschas, Jan-Tobias, Bernius** und natürlich unsere **Familien-Hundiiis** mit den süssen Ohrschläpperliiies.;)

# REFERENCES


1. Medzhitov, R., *Recognition of microorganisms and activation of the immune response.* Nature, 2007. **449**(7164): p. 819-26.

2. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Introduction to the immune system; Innate and adaptive immunity*, in *Basic immunology*. 2015, ELSEVIER: St. Louis, Missouri. p. 3-5.

3. Ramachandra, L., D. Simmons, and C.V. Harding, *MHC molecules and microbial antigen processing in phagosomes.* Curr Opin Immunol, 2009. **21**(1): p. 98-104.

4. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.

5. Dancey, J.T., K.A. Deubelbeiss, L.A. Harker, and C.A. Finch, *Neutrophil kinetics in man.* J Clin Invest, 1976. **58**(3): p. 705-15.

6. Lieschke, G.J., D. Grail, G. Hodgson, D. Metcalf, E. Stanley, C. Cheers, K.J. Fowler, S. Basu, Y.F. Zhan, and A.R. Dunn, *Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization.* Blood, 1994. **84**(6): p. 1737-46.

7. Cua, D.J. and C.M. Tato, *Innate IL-17-producing cells: the sentinels of the immune system.* Nat Rev Immunol, 2010. **10**(7): p. 479-89.

8. Summers, C., S.M. Rankin, A.M. Condliffe, N. Singh, A.M. Peters, and E.R. Chilvers, *Neutrophil kinetics in health and disease.* Trends Immunol, 2010. **31**(8): p. 318-24.

9. Pillay, J., I. den Braber, N. Vrisekoop, L.M. Kwast, R.J. de Boer, J.A. Borghans, K. Tesselaar, and L. Koenderman, *In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days.* Blood, 2010. **116**(4): p. 625-7.

10. Tak, T., K. Tesselaar, J. Pillay, J.A. Borghans, and L. Koenderman, *What's your age again? Determination of human neutrophil half-lives revisited.* J Leukoc Biol, 2013. **94**(4): p. 595-601.

11. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.

12. Amulic, B., C. Cazalet, G.L. Hayes, K.D. Metzler, and A. Zychlinsky, *Neutrophil function: from mechanisms to disease.* Annu Rev Immunol, 2012. **30**: p. 459-89.

13. DeLeo, F.R., J. Renee, S. McCormick, M. Nakamura, M. Apicella, J.P. Weiss, and W.M. Nauseef, *Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly.* J Clin Invest, 1998. **101**(2): p. 455-63.

14. Guthrie, L.A., L.C. McPhail, P.M. Henson, and R.B. Johnston, Jr., *Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme.* J Exp Med, 1984. **160**(6): p. 1656-71.

15. Brooks, A.C., K.J. Rickards, and F.M. Cunningham, *CXCL8 attenuates chemoattractant-induced equine neutrophil migration.* Vet Immunol Immunopathol, 2011. **139**(2-4): p. 141-7.

16. Lefebvre, J.S., S. Marleau, V. Milot, T. Levesque, S. Picard, N. Flamand, and P. Borgeat, *Toll-like receptor ligands induce polymorphonuclear leukocyte migration: key roles for leukotriene B4 and platelet-activating factor.* FASEB J, 2010. **24**(2): p. 637-47.

17. Segal, A.W., J. Dorling, and S. Coade, *Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies.* J Cell Biol, 1980. **85**(1): p. 42-59.

18. Henry, R.M., A.D. Hoppe, N. Joshi, and J.A. Swanson, *The uniformity of phagosome maturation in macrophages.* J Cell Biol, 2004. **164**(2): p. 185-94.

19. Thomas, C.J. and K. Schroder, *Pattern recognition receptor function in neutrophils.* Trends Immunol, 2013. **34**(7): p. 317-28.

20. Futosi, K., S. Fodor, and A. Mocsai, *Neutrophil cell surface receptors and their intracellular signal transduction pathways.* Int Immunopharmacol, 2013. **17**(3): p. 638-50.

21. Segal, A.W., M. Geisow, R. Garcia, A. Harper, and R. Miller, *The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH.* Nature, 1981. **290**(5805): p. 406-9.

22. Babior, B.M., *Phagocytes and oxidative stress.* Am J Med, 2000. **109**(1): p. 33-44.

23. El-Benna, J., P.M. Dang, M.A. Gougerot-Pocidalo, and C. Elbim, *Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses.* Arch Immunol Ther Exp (Warsz), 2005. **53**(3): p. 199-206.

24. Hampton, M.B., A.J. Kettle, and C.C. Winterbourn, *Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing.* Blood, 1998. **92**(9): p. 3007-17.

25. Nauseef, W.M., *How human neutrophils kill and degrade microbes: an integrated view.* Immunol Rev, 2007. **219**: p. 88-102.

26. Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D.S. Weiss, Y. Weinrauch, and A. Zychlinsky, *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.

27. Buchanan, J.T., A.J. Simpson, R.K. Aziz, G.Y. Liu, S.A. Kristian, M. Kotb, J. Feramisco, and V. Nizet, *DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps.* Curr Biol, 2006. **16**(4): p. 396-400.

28. Yipp, B.G. and P. Kubes, *NETosis: how vital is it?* Blood, 2013. **122**(16): p. 2784-94.

29. Borregaard, N., *Neutrophils, from marrow to microbes.* Immunity, 2010. **33**(5): p. 657-70.

30. Borregaard, N., O.E. Sorensen, and K. Theilgaard-Monch, *Neutrophil granules: a library of innate immunity proteins.* Trends Immunol, 2007. **28**(8): p. 340-5.

31. Faurschou, M. and N. Borregaard, *Neutrophil granules and secretory vesicles in inflammation.* Microbes Infect, 2003. **5**(14): p. 1317-27.

32. Borregaard, N. and J.B. Cowland, *Granules of the human neutrophilic polymorphonuclear leukocyte.* Blood, 1997. **89**(10): p. 3503-21.

33. Borregaard, N., M. Sehested, B.S. Nielsen, H. Sengelov, and L. Kjeldsen, *Biosynthesis of granule proteins in normal human bone marrow cells. Gelatinase is a marker of terminal neutrophil differentiation.* Blood, 1995. **85**(3): p. 812-7.

34. Bainton, D.F., J.L. Ullyot, and M.G. Farquhar, *The development of neutrophilic polymorphonuclear leukocytes in human bone marrow.* J Exp Med, 1971. **134**(4): p. 907-34.

35. Lominadze, G., D.W. Powell, G.C. Luerman, A.J. Link, R.A. Ward, and K.R. McLeish, *Proteomic analysis of human neutrophil granules.* Mol Cell Proteomics, 2005. **4**(10): p. 1503-21.

36. Rorvig, S., O. Ostergaard, N.H. Heegaard, and N. Borregaard, *Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors.* J Leukoc Biol, 2013. **94**(4): p. 711-21.

37. Bainton, D.F. and M.G. Farquhar, *Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells.* J Cell Biol, 1968. **39**(2): p. 299-317.

38. Bainton, D.F. and M.G. Farquhar, *Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. I. Histochemical staining of bone marrow smears.* J Cell Biol, 1968. **39**(2): p. 286-98.

39. Egesten, A., J. Breton-Gorius, J. Guichard, U. Gullberg, and I. Olsson, *The heterogeneity of azurophil granules in neutrophil promyelocytes: immunogold localization of myeloperoxidase, cathepsin G, elastase, proteinase 3, and bactericidal/permeability increasing protein.* Blood, 1994. **83**(10): p. 2985-94.

40. Ganz, T., M.E. Selsted, D. Szklarek, S.S. Harwig, K. Daher, D.F. Bainton, and R.I. Lehrer, *Defensins. Natural peptide antibiotics of human neutrophils.* J Clin Invest, 1985. **76**(4): p. 1427-35.

41. Smith, J.A., *Neutrophils, host defense, and inflammation: a double-edged sword.* J Leukoc Biol, 1994. **56**(6): p. 672-86.

42. Cassatella, M.A., *Neutrophils II*, in *Fundamentals of Inflammation*, C.N. Serhan, P.A. Ward, and D.W. Gilroy, Editors. 2010, Cambridge University Press: Cambridge University Press 2010. p. 49-64.

43. Johansson, L., A. Linner, J. Sunden-Cullberg, A. Haggar, H. Herwald, K. Lore, C.J. Treutiger, and A. Norrby-Teglund, *Neutrophil-derived hyperresistinemia in severe acute streptococcal infections.* J Immunol, 2009. **183**(6): p. 4047-54.

44. Kjeldsen, L., D.F. Bainton, H. Sengelov, and N. Borregaard, *Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils.* Blood, 1994. **83**(3): p. 799-807.

45. Sheshachalam, A., N. Srivastava, T. Mitchell, P. Lacy, and G. Eitzen, *Granule protein processing and regulated secretion in neutrophils.* Front Immunol, 2014. **5**: p. 448.

46. Lacy, P., *Mechanisms of degranulation in neutrophils.* Allergy Asthma Clin Immunol, 2006. **2**(3): p. 98-108.

47. Gautam, N., A.M. Olofsson, H. Herwald, L.F. Iversen, E. Lundgren-Akerlund, P. Hedqvist, K.E. Arfors, H. Flodgaard, and L. Lindbom, *Heparin-binding protein*

*(HBP/CAP37): a missing link in neutrophil-evoked alteration of vascular permeability.* Nat Med, 2001. **7**(10): p. 1123-7.

48. Linder, A., B. Christensson, H. Herwald, L. Bjorck, and P. Akesson, *Heparin-binding protein: an early marker of circulatory failure in sepsis.* Clin Infect Dis, 2009. **49**(7): p. 1044-50.

49. McCracken, J.M. and L.A. Allen, *Regulation of human neutrophil apoptosis and lifespan in health and disease.* J Cell Death, 2014. **7**: p. 15-23.

50. Greenlee-Wacker, M.C., *Clearance of apoptotic neutrophils and resolution of inflammation.* Immunol Rev, 2016. **273**(1): p. 357-70.

51. Bardoel, B.W., E.F. Kenny, G. Sollberger, and A. Zychlinsky, *The balancing act of neutrophils.* Cell Host Microbe, 2014. **15**(5): p. 526-36.

52. Holcomb, I.N., R.C. Kabakoff, B. Chan, T.W. Baker, A. Gurney, W. Henzel, C. Nelson, H.B. Lowman, B.D. Wright, N.J. Skelton, G.D. Frantz, D.B. Tumas, F.V. Peale, Jr., D.L. Shelton, and C.C. Hebert, *FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family.* EMBO J, 2000. **19**(15): p. 4046-55.

53. Kim, K.H., K. Lee, Y.S. Moon, and H.S. Sul, *A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation.* J Biol Chem, 2001. **276**(14): p. 11252-6.

54. Steppan, C.M., S.T. Bailey, S. Bhat, E.J. Brown, R.R. Banerjee, C.M. Wright, H.R. Patel, R.S. Ahima, and M.A. Lazar, *The hormone resistin links obesity to diabetes.* Nature, 2001. **409**(6818): p. 307-12.

55. Adeghate, E., *An update on the biology and physiology of resistin.* Cell Mol Life Sci, 2004. **61**(19-20): p. 2485-96.

56. Patel, L., A.C. Buckels, I.J. Kinghorn, P.R. Murdock, J.D. Holbrook, C. Plumpton, C.H. Macphee, and S.A. Smith, *Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators.* Biochem Biophys Res Commun, 2003. **300**(2): p. 472-6.

57. Bostrom, E.A., A. Tarkowski, and M. Bokarewa, *Resistin is stored in neutrophil granules being released upon challenge with inflammatory stimuli.* Biochim Biophys Acta, 2009. **1793**(12): p. 1894-900.

58. Lu, S.C., W.Y. Shieh, C.Y. Chen, S.C. Hsu, and H.L. Chen, *Lipopolysaccharide increases resistin gene expression in vivo and in vitro.* FEBS Lett, 2002. **530**(1-3): p. 158-62.

59. Minn, A.H., N.B. Patterson, S. Pack, S.C. Hoffmann, O. Gavrilova, C. Vinson, D.M. Harlan, and A. Shalev, *Resistin is expressed in pancreatic islets.* Biochem Biophys Res Commun, 2003. **310**(2): p. 641-5.

60. Yura, S., N. Sagawa, H. Itoh, K. Kakui, M.A. Nuamah, D. Korita, M. Takemura, and S. Fujii, *Resistin is expressed in the human placenta.* J Clin Endocrinol Metab, 2003. **88**(3): p. 1394-7.

61. Stejskal, D., J. Proskova, S. Adamovska, R. Jurakova, and J. Bartek, *Preliminary experience with resistin assessment in common population.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2002. **146**(2): p. 47-9.

62. Bokarewa, M., I. Nagaev, L. Dahlberg, U. Smith, and A. Tarkowski, *Resistin, an adipokine with potent proinflammatory properties.* J Immunol, 2005. **174**(9): p. 5789-95.

63. Bostrom, E.A., H.F. d'Elia, U. Dahlgren, C. Simark-Mattsson, B. Hasseus, H. Carlsten, A. Tarkowski, and M. Bokarewa, *Salivary resistin reflects local inflammation in Sjogren's syndrome.* J Rheumatol, 2008. **35**(10): p. 2005-11.

64. Lehrke, M., M.P. Reilly, S.C. Millington, N. Iqbal, D.J. Rader, and M.A. Lazar, *An inflammatory cascade leading to hyperresistinemia in humans.* PLoS Med, 2004. **1**(2): p. e45.

65. Nagaev, I., M. Bokarewa, A. Tarkowski, and U. Smith, *Human resistin is a systemic immune-derived proinflammatory cytokine targeting both leukocytes and adipocytes.* PLoS One, 2006. **1**: p. e31.

66. Pang, S.S. and Y.Y. Le, *Role of resistin in inflammation and inflammation-related diseases.* Cell Mol Immunol, 2006. **3**(1): p. 29-34.

67. McTernan, C.L., P.G. McTernan, A.L. Harte, P.L. Levick, A.H. Barnett, and S. Kumar, *Resistin, central obesity, and type 2 diabetes.* Lancet, 2002. **359**(9300): p. 46-7.

68. Savage, D.B., C.P. Sewter, E.S. Klenk, D.G. Segal, A. Vidal-Puig, R.V. Considine, and S. O'Rahilly, *Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans.* Diabetes, 2001. **50**(10): p. 2199-202.

69. Utzschneider, K.M., D.B. Carr, J. Tong, T.M. Wallace, R.L. Hull, S. Zraika, Q. Xiao, J.S. Mistry, B.M. Retzlaff, R.H. Knopp, and S.E. Kahn, *Resistin is not associated with insulin sensitivity or the metabolic syndrome in humans.* Diabetologia, 2005. **48**(11): p. 2330-3.

70. Silswal, N., A.K. Singh, B. Aruna, S. Mukhopadhyay, S. Ghosh, and N.Z. Ehtesham, *Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway.* Biochem Biophys Res Commun, 2005. **334**(4): p. 1092-101.

71. Kusminski, C.M., P.G. McTernan, and S. Kumar, *Role of resistin in obesity, insulin resistance and Type II diabetes.* Clin Sci (Lond), 2005. **109**(3): p. 243-56.

72. Lee, S., H.C. Lee, Y.W. Kwon, S.E. Lee, Y. Cho, J. Kim, S. Lee, J.Y. Kim, J. Lee, H.M. Yang, I. Mook-Jung, K.Y. Nam, J. Chung, M.A. Lazar, and H.S. Kim, *Adenylyl cyclase-associated protein 1 is a receptor for human resistin and mediates inflammatory actions of human monocytes.* Cell Metab, 2014. **19**(3): p. 484-97.

73. Hsu, W.Y., Y.W. Chao, Y.L. Tsai, C.C. Lien, C.F. Chang, M.C. Deng, L.T. Ho, C.F. Kwok, and C.C. Juan, *Resistin induces monocyte-endothelial cell adhesion by increasing ICAM-1 and VCAM-1 expression in endothelial cells via p38MAPK-dependent pathway.* J Cell Physiol, 2011. **226**(8): p. 2181-8.

74. Qiu, W., N. Chen, Q. Zhang, L. Zhuo, X. Wang, D. Wang, and H. Jin, *Resistin increases platelet P-selectin levels via p38 MAPK signal pathway.* Diab Vasc Dis Res, 2014. **11**(2): p. 121-4.

75. Manduteanu, I., M. Pirvulescu, A.M. Gan, D. Stan, V. Simion, E. Dragomir, M. Calin, A. Manea, and M. Simionescu, *Similar effects of resistin and high glucose*

*on P-selectin and fractalkine expression and monocyte adhesion in human endothelial cells.* Biochem Biophys Res Commun, 2010. **391**(3): p. 1443-8.

76. Migita, K., Y. Maeda, T. Miyashita, H. Kimura, M. Nakamura, H. Ishibashi, and K. Eguchi, *The serum levels of resistin in rheumatoid arthritis patients.* Clin Exp Rheumatol, 2006. **24**(6): p. 698-701.

77. Senolt, L., D. Housa, Z. Vernerova, T. Jirasek, R. Svobodova, D. Veigl, K. Anderlova, U. Muller-Ladner, K. Pavelka, and M. Haluzik, *Resistin in rheumatoid arthritis synovial tissue, synovial fluid and serum.* Ann Rheum Dis, 2007. **66**(4): p. 458-63.

78. Sunden-Cullberg, J., T. Nystrom, M.L. Lee, G.E. Mullins, L. Tokics, J. Andersson, A. Norrby-Teglund, and C.J. Treutiger, *Pronounced elevation of resistin correlates with severity of disease in severe sepsis and septic shock.* Crit Care Med, 2007. **35**(6): p. 1536-42.

79. Kosciuczuk, E.M., P. Lisowski, J. Jarczak, N. Strzalkowska, A. Jozwik, J. Horbanczuk, J. Krzyzewski, L. Zwierzchowski, and E. Bagnicka, *Cathelicidins: family of antimicrobial peptides. A review.* Mol Biol Rep, 2012. **39**(12): p. 10957-70.

80. Agerberth, B., H. Gunne, J. Odeberg, P. Kogner, H.G. Boman, and G.H. Gudmundsson, *FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis.* Proc Natl Acad Sci U S A, 1995. **92**(1): p. 195-9.

81. Cowland, J.B., A.H. Johnsen, and N. Borregaard, *hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules.* FEBS Lett, 1995. **368**(1): p. 173-6.

82. Larrick, J.W., M. Hirata, R.F. Balint, J. Lee, J. Zhong, and S.C. Wright, *Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein.* Infect Immun, 1995. **63**(4): p. 1291-7.

83. Durr, U.H., U.S. Sudheendra, and A. Ramamoorthy, *LL-37, the only human member of the cathelicidin family of antimicrobial peptides.* Biochim Biophys Acta, 2006. **1758**(9): p. 1408-25.

84. Xhindoli, D., S. Pacor, M. Benincasa, M. Scocchi, R. Gennaro, and A. Tossi, *The human cathelicidin LL-37--A pore-forming antibacterial peptide and host-cell modulator.* Biochim Biophys Acta, 2016. **1858**(3): p. 546-66.

85. Vandamme, D., B. Landuyt, W. Luyten, and L. Schoofs, *A comprehensive summary of LL-37, the factotum human cathelicidin peptide.* Cell Immunol, 2012. **280**(1): p. 22-35.

86. Bulow, E., N. Bengtsson, J. Calafat, U. Gullberg, and I. Olsson, *Sorting of neutrophil-specific granule protein human cathelicidin, hCAP-18, when constitutively expressed in myeloid cells.* J Leukoc Biol, 2002. **72**(1): p. 147-53.

87. Frohm, M., B. Agerberth, G. Ahangari, M. Stahle-Backdahl, S. Liden, H. Wigzell, and G.H. Gudmundsson, *The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders.* J Biol Chem, 1997. **272**(24): p. 15258-63.

88. Sorensen, O.E., P. Follin, A.H. Johnsen, J. Calafat, G.S. Tjabringa, P.S. Hiemstra, and N. Borregaard, *Human cathelicidin, hCAP-18, is processed to the*

*antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3.* Blood, 2001. **97**(12): p. 3951-9.

89. Henzler Wildman, K.A., D.K. Lee, and A. Ramamoorthy, *Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37.* Biochemistry, 2003. **42**(21): p. 6545-58.

90. Epand, R.M. and H.J. Vogel, *Diversity of antimicrobial peptides and their mechanisms of action.* Biochim Biophys Acta, 1999. **1462**(1-2): p. 11-28.

91. Henzler-Wildman, K.A., G.V. Martinez, M.F. Brown, and A. Ramamoorthy, *Perturbation of the hydrophobic core of lipid bilayers by the human antimicrobial peptide LL-37.* Biochemistry, 2004. **43**(26): p. 8459-69.

92. Lee, C.C., Y. Sun, S. Qian, and H.W. Huang, *Transmembrane pores formed by human antimicrobial peptide LL-37.* Biophys J, 2011. **100**(7): p. 1688-96.

93. Carapetis, J.R., A.C. Steer, E.K. Mulholland, and M. Weber, *The global burden of group A streptococcal diseases.* Lancet Infect Dis, 2005. **5**(11): p. 685-94.

94. Stevens, D.L., *Streptococcal toxic-shock syndrome: spectrum of disease, pathogenesis, and new concepts in treatment.* Emerg Infect Dis, 1995. **1**(3): p. 69-78.

95. Osterlund, A., R. Popa, T. Nikkila, A. Scheynius, and L. Engstrand, *Intracellular reservoir of Streptococcus pyogenes in vivo: a possible explanation for recurrent pharyngotonsillitis.* Laryngoscope, 1997. **107**(5): p. 640-7.

96. Cunningham, M.W., *Pathogenesis of group A streptococcal infections.* Clin Microbiol Rev, 2000. **13**(3): p. 470-511.

97. Cunningham, M.W., *Post-Streptococcal Autoimmune Sequelae: Rheumatic Fever and Beyond*, in *Streptococcus pyogenes : Basic Biology to Clinical Manifestations*, J.J. Ferretti, D.L. Stevens, and V.A. Fischetti, Editors. 2016: Oklahoma City (OK).

98. Lancefield, R.C., *The Antigenic Complex of Streptococcus Haemolyticus : I. Demonstration of a Type-Specific Substance in Extracts of Streptococcus Haemolyticus.* J Exp Med, 1928. **47**(1): p. 91-103.

99. Lancefield, R.C. and V.P. Dole, *The Properties of T Antigens Extracted from Group a Hemolytic Streptococci.* J Exp Med, 1946. **84**(5): p. 449-71.

100. Ward, H.K. and G. Rudd, *Studies on Haemolytic Streptococci from Human Sources. I. The Cultural Characteristics of Potentially Virulent Strains.* Australian Journal of Experimental Biology and Medical Science, 1938. **16**: p. 181-92.

101. Beall, B., R. Facklam, and T. Thompson, *Sequencing emm-specific PCR products for routine and accurate typing of group A streptococci.* J Clin Microbiol, 1996. **34**(4): p. 953-8.

102. Metzgar, D. and A. Zampolli, *The M protein of group A Streptococcus is a key virulence factor and a clinically relevant strain identification marker.* Virulence, 2011. **2**(5): p. 402-12.

103. Bisno, A.L., M.O. Brito, and C.M. Collins, *Molecular basis of group A streptococcal virulence.* Lancet Infect Dis, 2003. **3**(4): p. 191-200.

104. Wessels, M.R., A.E. Moses, J.B. Goldberg, and T.J. DiCesare, *Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci.* Proc Natl Acad Sci U S A, 1991. **88**(19): p. 8317-21.

105. Bisno, A.L., *Alternate complement pathway activation by group A streptococci: role of M-protein.* Infect Immun, 1979. **26**(3): p. 1172-6.

106. Horstmann, R.D., H.J. Sievertsen, J. Knobloch, and V.A. Fischetti, *Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H.* Proc Natl Acad Sci U S A, 1988. **85**(5): p. 1657-61.

107. Ji, Y., N. Schnitzler, E. DeMaster, and P. Cleary, *Impact of M49, Mrp, Enn, and C5a peptidase proteins on colonization of the mouse oral mucosa by Streptococcus pyogenes.* Infect Immun, 1998. **66**(11): p. 5399-405.

108. Podbielski, A., N. Schnitzler, P. Beyhs, and M.D. Boyle, *M-related protein (Mrp) contributes to group A streptococcal resistance to phagocytosis by human granulocytes.* Mol Microbiol, 1996. **19**(3): p. 429-41.

109. Wexler, D.E., D.E. Chenoweth, and P.P. Cleary, *Mechanism of action of the group A streptococcal C5a inactivator.* Proc Natl Acad Sci U S A, 1985. **82**(23): p. 8144-8.

110. Okada, N., A.P. Pentland, P. Falk, and M.G. Caparon, *M protein and protein F act as important determinants of cell-specific tropism of Streptococcus pyogenes in skin tissue.* J Clin Invest, 1994. **94**(3): p. 965-77.

111. Bober, M., M. Morgelin, A.I. Olin, U. von Pawel-Rammingen, and M. Collin, *The membrane bound LRR lipoprotein Slr, and the cell wall-anchored M1 protein from Streptococcus pyogenes both interact with type I collagen.* PLoS One, 2011. **6**(5): p. e20345.

112. Dinkla, K., M. Rohde, W.T. Jansen, J.R. Carapetis, G.S. Chhatwal, and S.R. Talay, *Streptococcus pyogenes recruits collagen via surface-bound fibronectin: a novel colonization and immune evasion mechanism.* Mol Microbiol, 2003. **47**(3): p. 861-9.

113. Dinkla, K., M. Rohde, W.T. Jansen, E.L. Kaplan, G.S. Chhatwal, and S.R. Talay, *Rheumatic fever-associated Streptococcus pyogenes isolates aggregate collagen.* J Clin Invest, 2003. **111**(12): p. 1905-12.

114. Terao, Y., *The virulence factors and pathogenic mechanisms of Streptococcus pyogenes.* Journal of Oral Biosciences, 2012. **54**(2): p. 96-100.

115. Johnsson, E., A. Thern, B. Dahlback, L.O. Heden, M. Wikstrom, and G. Lindahl, *A highly variable region in members of the streptococcal M protein family binds the human complement regulator C4BP.* J Immunol, 1996. **157**(7): p. 3021-9.

116. Kotarsky, H., J. Hellwage, E. Johnsson, C. Skerka, H.G. Svensson, G. Lindahl, U. Sjobring, and P.F. Zipfel, *Identification of a domain in human factor H and factor H-like protein-1 required for the interaction with streptococcal M proteins.* J Immunol, 1998. **160**(7): p. 3349-54.

117. Smeesters, P.R., D.J. McMillan, and K.S. Sriprakash, *The streptococcal M protein: a highly versatile molecule.* Trends Microbiol, 2010. **18**(6): p. 275-82.

118. Brouwer, S., T.C. Barnett, T. Rivera-Hernandez, M. Rohde, and M.J. Walker, *Streptococcus pyogenes adhesion and colonization.* FEBS Lett, 2016. **590**(21): p. 3739-3757.

119. Herwald, H., H. Cramer, M. Morgelin, W. Russell, U. Sollenberg, A. Norrby-Teglund, H. Flodgaard, L. Lindbom, and L. Bjorck, *M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage.* Cell, 2004. **116**(3): p. 367-79.

120. Pahlman, L.I., M. Morgelin, J. Eckert, L. Johansson, W. Russell, K. Riesbeck, O. Soehnlein, L. Lindbom, A. Norrby-Teglund, R.R. Schumann, L. Bjorck, and H. Herwald, *Streptococcal M protein: a multipotent and powerful inducer of inflammation.* J Immunol, 2006. **177**(2): p. 1221-8.

121. Pahlman, L.I., A.I. Olin, J. Darenberg, M. Morgelin, M. Kotb, H. Herwald, and A. Norrby-Teglund, *Soluble M1 protein of Streptococcus pyogenes triggers potent T cell activation.* Cell Microbiol, 2008. **10**(2): p. 404-14.

122. Cue, D., P.E. Dombek, H. Lam, and P.P. Cleary, *Streptococcus pyogenes serotype M1 encodes multiple pathways for entry into human epithelial cells.* Infect Immun, 1998. **66**(10): p. 4593-601.

123. Dombek, P.E., D. Cue, J. Sedgewick, H. Lam, S. Ruschkowski, B.B. Finlay, and P.P. Cleary, *High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements.* Mol Microbiol, 1999. **31**(3): p. 859-70.

124. Nobbs, A.H., R.J. Lamont, and H.F. Jenkinson, *Streptococcus adherence and colonization.* Microbiol Mol Biol Rev, 2009. **73**(3): p. 407-50, Table of Contents.

125. Bisno, A.L. and D.L. Stevens, *Streptococcal infections of skin and soft tissues.* N Engl J Med, 1996. **334**(4): p. 240-5.

126. Starr, C.R. and N.C. Engleberg, *Role of hyaluronidase in subcutaneous spread and growth of group A streptococcus.* Infect Immun, 2006. **74**(1): p. 40-8.

127. Sun, H., U. Ringdahl, J.W. Homeister, W.P. Fay, N.C. Engleberg, A.Y. Yang, L.S. Rozek, X. Wang, U. Sjobring, and D. Ginsburg, *Plasminogen is a critical host pathogenicity factor for group A streptococcal infection.* Science, 2004. **305**(5688): p. 1283-6.

128. Collin, M. and A. Olsen, *Effect of SpeB and EndoS from Streptococcus pyogenes on human immunoglobulins.* Infect Immun, 2001. **69**(11): p. 7187-9.

129. Egesten, A., A.I. Olin, H.M. Linge, M. Yadav, M. Morgelin, A. Karlsson, and M. Collin, *SpeB of Streptococcus pyogenes differentially modulates antibacterial and receptor activating properties of human chemokines.* PLoS One, 2009. **4**(3): p. e4769.

130. Elliott, S.D., *A Proteolytic Enzyme Produced by Group a Streptococci with Special Reference to Its Effect on the Type-Specific M Antigen.* J Exp Med, 1945. **81**(6): p. 573-92.

131. Matsuka, Y.V., S. Pillai, S. Gubba, J.M. Musser, and S.B. Olmsted, *Fibrinogen cleavage by the Streptococcus pyogenes extracellular cysteine protease and generation of antibodies that inhibit enzyme proteolytic activity.* Infect Immun, 1999. **67**(9): p. 4326-33.

132. Cole, J.N., J.D. McArthur, F.C. McKay, M.L. Sanderson-Smith, A.J. Cork, M. Ranson, M. Rohde, A. Itzek, H. Sun, D. Ginsburg, M. Kotb, V. Nizet, G.S. Chhatwal, and M.J. Walker, *Trigger for group A streptococcal M1T1 invasive disease.* FASEB J, 2006. **20**(10): p. 1745-7.

133. Nyberg, P., M. Rasmussen, and L. Bjorck, *alpha2-Macroglobulin-proteinase complexes protect Streptococcus pyogenes from killing by the antimicrobial peptide LL-37.* J Biol Chem, 2004. **279**(51): p. 52820-3.

134. Schmidtchen, A., I.M. Frick, E. Andersson, H. Tapper, and L. Bjorck, *Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37.* Mol Microbiol, 2002. **46**(1): p. 157-68.

135. Nyberg, P., M. Rasmussen, U. Von Pawel-Rammingen, and L. Bjorck, *SpeB modulates fibronectin-dependent internalization of Streptococcus pyogenes by efficient proteolysis of cell-wall-anchored protein F1.* Microbiology, 2004. **150**(Pt 5): p. 1559-69.

136. Svensson, M.D., U. Sjobring, F. Luo, and D.E. Bessen, *Roles of the plasminogen activator streptokinase and the plasminogen-associated M protein in an experimental model for streptococcal impetigo.* Microbiology, 2002. **148**(Pt 12): p. 3933-45.

137. Walker, M.J., A. Hollands, M.L. Sanderson-Smith, J.N. Cole, J.K. Kirk, A. Henningham, J.D. McArthur, K. Dinkla, R.K. Aziz, R.G. Kansal, A.J. Simpson, J.T. Buchanan, G.S. Chhatwal, M. Kotb, and V. Nizet, *DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection.* Nat Med, 2007. **13**(8): p. 981-5.

138. Sumby, P., K.D. Barbian, D.J. Gardner, A.R. Whitney, D.M. Welty, R.D. Long, J.R. Bailey, M.J. Parnell, N.P. Hoe, G.G. Adams, F.R. Deleo, and J.M. Musser, *Extracellular deoxyribonuclease made by group A Streptococcus assists pathogenesis by enhancing evasion of the innate immune response.* Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1679-84.

139. Bhakdi, S., J. Tranum-Jensen, and A. Sziegoleit, *Mechanism of membrane damage by streptolysin-O.* Infect Immun, 1985. **47**(1): p. 52-60.

140. Higashi, D.L., N. Biais, D.L. Donahue, J.A. Mayfield, C.R. Tessier, K. Rodriguez, B.L. Ashfeld, J. Luchetti, V.A. Ploplis, F.J. Castellino, and S.W. Lee, *Activation of band 3 mediates group A Streptococcus streptolysin S-based beta-haemolysis.* Nat Microbiol, 2016. **1**: p. 15004.

141. Sloan, D.C. and R.J. Haslam, *Protein kinase C-dependent and Ca2+-dependent mechanisms of secretion from streptolysin O-permeabilized platelets: effects of leakage of cytosolic proteins.* Biochem J, 1997. **328 ( Pt 1)**: p. 13-21.

142. Tweten, R.K., *Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins.* Infect Immun, 2005. **73**(10): p. 6199-209.

143. Bhakdi, S. and J. Tranum-Jensen, *Membrane damage by channel-forming proteins: staphylococcal alpha-toxin, streptolysin-O and the C5b-9 complement complex.* Biochem Soc Symp, 1985. **50**: p. 221-33.

144. Ato, M., T. Ikebe, H. Kawabata, T. Takemori, and H. Watanabe, *Incompetence of neutrophils to invasive group A streptococcus is attributed to induction of plural virulence factors by dysfunction of a regulator.* PLoS One, 2008. **3**(10): p. e3455.

145. Uchiyama, S., S. Dohrmann, A.M. Timmer, N. Dixit, M. Ghochani, T. Bhandari, J.C. Timmer, K. Sprague, J. Bubeck-Wardenburg, S.I. Simon, and V. Nizet, *Streptolysin O Rapidly Impairs Neutrophil Oxidative Burst and Antibacterial Responses to Group A Streptococcus.* Front Immunol, 2015. **6**: p. 581.

146. Bryant, A.E., C.R. Bayer, R.Y. Chen, P.H. Guth, R.J. Wallace, and D.L. Stevens, *Vascular dysfunction and ischemic destruction of tissue in Streptococcus pyogenes infection: the role of streptolysin O-induced platelet/neutrophil complexes.* J Infect Dis, 2005. **192**(6): p. 1014-22.

147. Nilsson, M., O.E. Sorensen, M. Morgelin, M. Weineisen, U. Sjobring, and H. Herwald, *Activation of human polymorphonuclear neutrophils by streptolysin O from Streptococcus pyogenes leads to the release of proinflammatory mediators.* Thromb Haemost, 2006. **95**(6): p. 982-90.

148. Kotb, M., A. Norrby-Teglund, A. McGeer, H. El-Sherbini, M.T. Dorak, A. Khurshid, K. Green, J. Peeples, J. Wade, G. Thomson, B. Schwartz, and D.E. Low, *An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections.* Nat Med, 2002. **8**(12): p. 1398-404.

149. Norrby-Teglund, A., P. Thulin, B.S. Gan, M. Kotb, A. McGeer, J. Andersson, and D.E. Low, *Evidence for superantigen involvement in severe group a streptococcal tissue infections.* J Infect Dis, 2001. **184**(7): p. 853-60.

150. Stevens, D.L., *Invasive group A streptococcus infections.* Clin Infect Dis, 1992. **14**(1): p. 2-11.

151. Kotb, M., *Bacterial pyrogenic exotoxins as superantigens.* Clin Microbiol Rev, 1995. **8**(3): p. 411-26.

152. Manders, S.M., *Toxin-mediated streptococcal and staphylococcal disease.* J Am Acad Dermatol, 1998. **39**(3): p. 383-98; quiz 399-400.

153. Cohen, J., *Shock factor shed by microbe.* Nat Med, 2004. **10**(4): p. 342-3.

154. Gorwitz, R.J., D. Kruszon-Moran, S.K. McAllister, G. McQuillan, L.K. McDougal, G.E. Fosheim, B.J. Jensen, G. Killgore, F.C. Tenover, and M.J. Kuehnert, *Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004.* J Infect Dis, 2008. **197**(9): p. 1226-34.

155. Kluytmans, J., A. van Belkum, and H. Verbrugh, *Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks.* Clin Microbiol Rev, 1997. **10**(3): p. 505-20.

156. Hartman, B.J. and A. Tomasz, *Low-affinity penicillin-binding protein associated with beta-lactam resistance in Staphylococcus aureus.* J Bacteriol, 1984. **158**(2): p. 513-6.

157. Ubukata, K., R. Nonoguchi, M. Matsuhashi, and M. Konno, *Expression and inducibility in Staphylococcus aureus of the mecA gene, which encodes a methicillin-resistant S. aureus-specific penicillin-binding protein.* J Bacteriol, 1989. **171**(5): p. 2882-5.

158. Zetola, N., J.S. Francis, E.L. Nuermberger, and W.R. Bishai, *Community-acquired meticillin-resistant Staphylococcus aureus: an emerging threat.* Lancet Infect Dis, 2005. **5**(5): p. 275-86.

159. David, M.Z. and R.S. Daum, *Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic.* Clin Microbiol Rev, 2010. **23**(3): p. 616-87.

160. Lowy, F.D., *Staphylococcus aureus infections.* N Engl J Med, 1998. **339**(8): p. 520-32.

161. Wenzel, R.P. and T.M. Perl, *The significance of nasal carriage of Staphylococcus aureus and the incidence of postoperative wound infection.* J Hosp Infect, 1995. **31**(1): p. 13-24.

162. Klevens, R.M., M.A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L.H. Harrison, R. Lynfield, G. Dumyati, J.M. Townes, A.S. Craig, E.R. Zell, G.E. Fosheim, L.K. McDougal, R.B. Carey, S.K. Fridkin, and M.I. Active Bacterial Core surveillance, *Invasive methicillin-resistant Staphylococcus aureus infections in the United States.* JAMA, 2007. **298**(15): p. 1763-71.

163. Noskin, G.A., R.J. Rubin, J.J. Schentag, J. Kluytmans, E.C. Hedblom, C. Jacobson, M. Smulders, E. Gemmen, and M. Bharmal, *National trends in Staphylococcus aureus infection rates: impact on economic burden and mortality over a 6-year period (1998-2003).* Clin Infect Dis, 2007. **45**(9): p. 1132-40.

164. Otter, J.A. and G.L. French, *Molecular epidemiology of community-associated meticillin-resistant Staphylococcus aureus in Europe.* Lancet Infect Dis, 2010. **10**(4): p. 227-39.

165. Takano, T., W. Higuchi, and T. Yamamoto, *Superior in vitro activity of carbapenems over anti-methicillin-resistant Staphylococcus aureus (MRSA) and some related antimicrobial agents for community-acquired MRSA but not for hospital-acquired MRSA.* J Infect Chemother, 2009. **15**(1): p. 54-7.

166. Foster, T.J., *Immune evasion by staphylococci.* Nat Rev Microbiol, 2005. **3**(12): p. 948-58.

167. Thammavongsa, V., H.K. Kim, D. Missiakas, and O. Schneewind, *Staphylococcal manipulation of host immune responses.* Nat Rev Microbiol, 2015. **13**(9): p. 529-43.

168. Alonzo, F., 3rd and V.J. Torres, *The bicomponent pore-forming leucocidins of Staphylococcus aureus.* Microbiol Mol Biol Rev, 2014. **78**(2): p. 199-230.

169. Woodin, A.M., *Purification of the two components of leucocidin from Staphylococcus aureus.* Biochem J, 1960. **75**: p. 158-65.

170. Colin, D.A., I. Mazurier, S. Sire, and V. Finck-Barbancon, *Interaction of the two components of leukocidin from Staphylococcus aureus with human polymorphonuclear leukocyte membranes: sequential binding and subsequent activation.* Infect Immun, 1994. **62**(8): p. 3184-8.

171. Aman, M.J., H. Karauzum, M.G. Bowden, and T.L. Nguyen, *Structural model of the pre-pore ring-like structure of Panton-Valentine leukocidin: providing dimensionality to biophysical and mutational data.* J Biomol Struct Dyn, 2010. **28**(1): p. 1-12.

172. Yamashita, K., Y. Kawai, Y. Tanaka, N. Hirano, J. Kaneko, N. Tomita, M. Ohta, Y. Kamio, M. Yao, and I. Tanaka, *Crystal structure of the octameric pore of staphylococcal gamma-hemolysin reveals the beta-barrel pore formation*

*mechanism by two components.* Proc Natl Acad Sci U S A, 2011. **108**(42): p. 17314-9.

173. Berube, B.J. and J. Bubeck Wardenburg, *Staphylococcus aureus alpha-toxin: nearly a century of intrigue.* Toxins (Basel), 2013. **5**(6): p. 1140-66.

174. Song, L., M.R. Hobaugh, C. Shustak, S. Cheley, H. Bayley, and J.E. Gouaux, *Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore.* Science, 1996. **274**(5294): p. 1859-66.

175. DuMont, A.L. and V.J. Torres, *Cell targeting by the Staphylococcus aureus pore-forming toxins: it's not just about lipids.* Trends Microbiol, 2014. **22**(1): p. 21-7.

176. Yoong, P. and V.J. Torres, *The effects of Staphylococcus aureus leukotoxins on the host: cell lysis and beyond.* Curr Opin Microbiol, 2013. **16**(1): p. 63-9.

177. Dumont, A.L., T.K. Nygaard, R.L. Watkins, A. Smith, L. Kozhaya, B.N. Kreiswirth, B. Shopsin, D. Unutmaz, J.M. Voyich, and V.J. Torres, *Characterization of a new cytotoxin that contributes to Staphylococcus aureus pathogenesis.* Mol Microbiol, 2011. **79**(3): p. 814-25.

178. DuMont, A.L., P. Yoong, C.J. Day, F. Alonzo, 3rd, W.H. McDonald, M.P. Jennings, and V.J. Torres, *Staphylococcus aureus LukAB cytotoxin kills human neutrophils by targeting the CD11b subunit of the integrin Mac-1.* Proc Natl Acad Sci U S A, 2013. **110**(26): p. 10794-9.

179. Gillet, Y., B. Issartel, P. Vanhems, J.C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piemont, N. Brousse, D. Floret, and J. Etienne, *Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients.* Lancet, 2002. **359**(9308): p. 753-9.

180. Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M.O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne, *Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia.* Clin Infect Dis, 1999. **29**(5): p. 1128-32.

181. Loffler, B., M. Hussain, M. Grundmeier, M. Bruck, D. Holzinger, G. Varga, J. Roth, B.C. Kahl, R.A. Proctor, and G. Peters, *Staphylococcus aureus panton-valentine leukocidin is a very potent cytotoxic factor for human neutrophils.* PLoS Pathog, 2010. **6**(1): p. e1000715.

182. Spaan, A.N., T. Henry, W.J. van Rooijen, M. Perret, C. Badiou, P.C. Aerts, J. Kemmink, C.J. de Haas, K.P. van Kessel, F. Vandenesch, G. Lina, and J.A. van Strijp, *The staphylococcal toxin Panton-Valentine Leukocidin targets human C5a receptors.* Cell Host Microbe, 2013. **13**(5): p. 584-94.

183. Graves, S.F., S.D. Kobayashi, K.R. Braughton, A.R. Whitney, D.E. Sturdevant, D.L. Rasmussen, L.N. Kirpotina, M.T. Quinn, and F.R. DeLeo, *Sublytic concentrations of Staphylococcus aureus Panton-Valentine leukocidin alter human PMN gene expression and enhance bactericidal capacity.* J Leukoc Biol, 2012. **92**(2): p. 361-74.

184. Kamio, Y., A. Rahman, H. Nariya, T. Ozawa, and K. Izaki, *The two Staphylococcal bi-component toxins, leukocidin and gamma-hemolysin, share one component in common.* FEBS Lett, 1993. **321**(1): p. 15-8.

185. von Eiff, C., A.W. Friedrich, G. Peters, and K. Becker, *Prevalence of genes encoding for members of the staphylococcal leukotoxin family among clinical isolates of Staphylococcus aureus.* Diagn Microbiol Infect Dis, 2004. **49**(3): p. 157-62.

186. Malachowa, N., A.R. Whitney, S.D. Kobayashi, D.E. Sturdevant, A.D. Kennedy, K.R. Braughton, D.W. Shabb, B.A. Diep, H.F. Chambers, M. Otto, and F.R. DeLeo, *Global changes in Staphylococcus aureus gene expression in human blood.* PLoS One, 2011. **6**(4): p. e18617.

187. Spaan, A.N., M. Vrieling, P. Wallet, C. Badiou, T. Reyes-Robles, E.A. Ohneck, Y. Benito, C.J. de Haas, C.J. Day, M.P. Jennings, G. Lina, F. Vandenesch, K.P. van Kessel, V.J. Torres, J.A. van Strijp, and T. Henry, *The staphylococcal toxins gamma-haemolysin AB and CB differentially target phagocytes by employing specific chemokine receptors.* Nat Commun, 2014. **5**: p. 5438.

188. McCarthy, A.J. and J.A. Lindsay, *Staphylococcus aureus innate immune evasion is lineage-specific: a bioinfomatics study.* Infect Genet Evol, 2013. **19**: p. 7-14.

189. Alonzo, F., 3rd, L. Kozhaya, S.A. Rawlings, T. Reyes-Robles, A.L. DuMont, D.G. Myszka, N.R. Landau, D. Unutmaz, and V.J. Torres, *CCR5 is a receptor for Staphylococcus aureus leukotoxin ED.* Nature, 2013. **493**(7430): p. 51-5.

190. Reyes-Robles, T., F. Alonzo, 3rd, L. Kozhaya, D.B. Lacy, D. Unutmaz, and V.J. Torres, *Staphylococcus aureus leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection.* Cell Host Microbe, 2013. **14**(4): p. 453-9.

191. Ventura, C.L., N. Malachowa, C.H. Hammer, G.A. Nardone, M.A. Robinson, S.D. Kobayashi, and F.R. DeLeo, *Identification of a novel Staphylococcus aureus two-component leukotoxin using cell surface proteomics.* PLoS One, 2010. **5**(7): p. e11634.

192. Malachowa, N., S.D. Kobayashi, B. Freedman, D.W. Dorward, and F.R. DeLeo, *Staphylococcus aureus leukotoxin GH promotes formation of neutrophil extracellular traps.* J Immunol, 2013. **191**(12): p. 6022-9.

193. Okumura, C.Y. and V. Nizet, *Subterfuge and sabotage: evasion of host innate defenses by invasive gram-positive bacterial pathogens.* Annu Rev Microbiol, 2014. **68**: p. 439-58.

194. Schmidt, K.H., K. Mann, J. Cooney, and W. Kohler, *Multiple binding of type 3 streptococcal M protein to human fibrinogen, albumin and fibronectin.* FEMS Immunol Med Microbiol, 1993. **7**(2): p. 135-43.

195. Jonsson, K., C. Signas, H.P. Muller, and M. Lindberg, *Two different genes encode fibronectin binding proteins in Staphylococcus aureus. The complete nucleotide sequence and characterization of the second gene.* Eur J Biochem, 1991. **202**(3): p. 1041-8.

196. Hair, P.S., C.G. Echague, A.M. Sholl, J.A. Watkins, J.A. Geoghegan, T.J. Foster, and K.M. Cunnion, *Clumping factor A interaction with complement factor I increases C3b cleavage on the bacterial surface of Staphylococcus aureus and decreases complement-mediated phagocytosis.* Infect Immun, 2010. **78**(4): p. 1717-27.

197. Carlsson, F., C. Sandin, and G. Lindahl, *Human fibrinogen bound to Streptococcus pyogenes M protein inhibits complement deposition via the classical pathway.* Mol Microbiol, 2005. **56**(1): p. 28-39.

198. Collin, M., M.D. Svensson, A.G. Sjoholm, J.C. Jensenius, U. Sjobring, and A. Olsen, *EndoS and SpeB from Streptococcus pyogenes inhibit immunoglobulin-mediated opsonophagocytosis.* Infect Immun, 2002. **70**(12): p. 6646-51.

199. Honda-Ogawa, M., T. Ogawa, Y. Terao, T. Sumitomo, M. Nakata, K. Ikebe, Y. Maeda, and S. Kawabata, *Cysteine proteinase from Streptococcus pyogenes enables evasion of innate immunity via degradation of complement factors.* J Biol Chem, 2013. **288**(22): p. 15854-64.

200. Jusko, M., J. Potempa, T. Kantyka, E. Bielecka, H.K. Miller, M. Kalinska, G. Dubin, P. Garred, L.N. Shaw, and A.M. Blom, *Staphylococcal proteases aid in evasion of the human complement system.* J Innate Immun, 2014. **6**(1): p. 31-46.

201. Kim, H.K., V. Thammavongsa, O. Schneewind, and D. Missiakas, *Recurrent infections and immune evasion strategies of Staphylococcus aureus.* Curr Opin Microbiol, 2012. **15**(1): p. 92-9.

202. Johansson, L., P. Thulin, P. Sendi, E. Hertzen, A. Linder, P. Akesson, D.E. Low, B. Agerberth, and A. Norrby-Teglund, *Cathelicidin LL-37 in severe Streptococcus pyogenes soft tissue infections in humans.* Infect Immun, 2008. **76**(8): p. 3399-404.

203. Sieprawska-Lupa, M., P. Mydel, K. Krawczyk, K. Wojcik, M. Puklo, B. Lupa, P. Suder, J. Silberring, M. Reed, J. Pohl, W. Shafer, F. McAleese, T. Foster, J. Travis, and J. Potempa, *Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases.* Antimicrob Agents Chemother, 2004. **48**(12): p. 4673-9.

204. Berends, E.T., A.R. Horswill, N.M. Haste, M. Monestier, V. Nizet, and M. von Kockritz-Blickwede, *Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps.* J Innate Immun, 2010. **2**(6): p. 576-86.

205. Thulin, P., L. Johansson, D.E. Low, B.S. Gan, M. Kotb, A. McGeer, and A. Norrby-Teglund, *Viable group A streptococci in macrophages during acute soft tissue infection.* PLoS Med, 2006. **3**(3): p. e53.

206. Rosch, J.W. and M.G. Caparon, *The ExPortal: an organelle dedicated to the biogenesis of secreted proteins in Streptococcus pyogenes.* Mol Microbiol, 2005. **58**(4): p. 959-68.

207. Rosch, J.W., F.F. Hsu, and M.G. Caparon, *Anionic lipids enriched at the ExPortal of Streptococcus pyogenes.* J Bacteriol, 2007. **189**(3): p. 801-6.

208. Vega, L.A. and M.G. Caparon, *Cationic antimicrobial peptides disrupt the Streptococcus pyogenes ExPortal.* Mol Microbiol, 2012. **85**(6): p. 1119-32.

209. Scheiermann, C., Y. Kunisaki, and P.S. Frenette, *Circadian control of the immune system.* Nat Rev Immunol, 2013. **13**(3): p. 190-8.

210. Haus, E. and M.H. Smolensky, *Biologic rhythms in the immune system.* Chronobiol Int, 1999. **16**(5): p. 581-622.

211. Collins, S.J., *The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression.* Blood, 1987. **70**(5): p. 1233-44.

212. Verstuyf, A., C. Mathieu, L. Verlinden, M. Waer, B.K. Tan, and R. Bouillon, *Differentiation induction of human leukemia cells (HL60) by a combination of 1,25-dihydroxyvitamin D3 and retinoic acid (all trans or 9-cis).* J Steroid Biochem Mol Biol, 1995. **53**(1-6): p. 431-41.

213. Nordenfelt, P., S. Bauer, P. Lonnbro, and H. Tapper, *Phagocytosis of Streptococcus pyogenes by all-trans retinoic acid-differentiated HL-60 cells: roles of azurophilic granules and NADPH oxidase.* PLoS One, 2009. **4**(10): p. e7363.

214. Luca-Harari, B., J. Darenberg, S. Neal, T. Siljander, L. Strakova, A. Tanna, R. Creti, K. Ekelund, M. Koliou, P.T. Tassios, M. van der Linden, M. Straut, J. Vuopio-Varkila, A. Bouvet, A. Efstratiou, C. Schalen, B. Henriques-Normark, E.S.G. Strep, and A. Jasir, *Clinical and microbiological characteristics of severe Streptococcus pyogenes disease in Europe.* J Clin Microbiol, 2009. **47**(4): p. 1155-65.

215. Siemens, N., B. Chakrakodi, S.M. Shambat, M. Morgan, H. Bergsten, O. Hyldegaard, S. Skrede, P. Arnell, M.B. Madsen, and L. Johansson, *Biofilm in group A streptococcal necrotizing soft tissue infections.* JCI Insight, 2016. **1**(10): p. e87882.

216. Nelson, D.C., J. Garbe, and M. Collin, *Cysteine proteinase SpeB from Streptococcus pyogenes - a potent modifier of immunologically important host and bacterial proteins.* Biol Chem, 2011. **392**(12): p. 1077-88.

217. Graham, M.R., L.M. Smoot, C.A. Migliaccio, K. Virtaneva, D.E. Sturdevant, S.F. Porcella, M.J. Federle, G.J. Adams, J.R. Scott, and J.M. Musser, *Virulence control in group A Streptococcus by a two-component gene regulatory system: global expression profiling and in vivo infection modeling.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13855-60.

218. Vega, L.A., H. Malke, and K.S. McIver, *Virulence-Related Transcriptional Regulators of Streptococcus pyogenes*, in *Streptococcus pyogenes: Basic Biology to Clinical Manifestations*, J.J. Ferretti, D.L. Stevens, and V.A. Fischetti, Editors. 2016, University of Oklahoma Health Sciences Center

(c) The University of Oklahoma Health Sciences Center.: Oklahoma City (OK).

219. Kansal, R.G., V. Datta, R.K. Aziz, N.F. Abdeltawab, S. Rowe, and M. Kotb, *Dissection of the molecular basis for hypervirulence of an in vivo-selected phenotype of the widely disseminated M1T1 strain of group A Streptococcus bacteria.* J Infect Dis, 2010. **201**(6): p. 855-65.

220. Cole, J.N., M.A. Pence, M. von Kockritz-Blickwede, A. Hollands, R.L. Gallo, M.J. Walker, and V. Nizet, *M protein and hyaluronic acid capsule are essential for in vivo selection of covRS mutations characteristic of invasive serotype M1T1 group A Streptococcus.* MBio, 2010. **1**(4).

221. Li, J., G. Liu, W. Feng, Y. Zhou, M. Liu, J.A. Wiley, and B. Lei, *Neutrophils select hypervirulent CovRS mutants of M1T1 group A Streptococcus during subcutaneous infection of mice.* Infect Immun, 2014. **82**(4): p. 1579-90.

222. Kansal, R.G., A. McGeer, D.E. Low, A. Norrby-Teglund, and M. Kotb, *Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases.* Infect Immun, 2000. **68**(11): p. 6362-9.

223. Olsen, R.J., A. Raghuram, C. Cantu, M.H. Hartman, F.E. Jimenez, S. Lee, A. Ngo, K.A. Rice, D. Saddington, H. Spillman, C. Valson, A.R. Flores, S.B. Beres, S.W. Long, W. Nasser, and J.M. Musser, *The majority of 9,729 group A streptococcus strains causing disease secrete SpeB cysteine protease: pathogenesis implications.* Infect Immun, 2015. **83**(12): p. 4750-8.

224. Rohde, M. and P.P. Cleary, *Adhesion and invasion of Streptococcus pyogenes into host cells and clinical relevance of intracellular streptococci*, in *Streptococcus pyogenes: Basic Biology to Clinical Manifestations*, J.J. Ferretti, D.L. Stevens, and V.A. Fischetti, Editors. 2016: Oklahoma City (OK).

225. Boone, T.J., C.A. Burnham, and G.J. Tyrrell, *Binding of group B streptococcal phosphoglycerate kinase to plasminogen and actin.* Microb Pathog, 2011. **51**(4): p. 255-61.

226. Boone, T.J. and G.J. Tyrrell, *Identification of the actin and plasminogen binding regions of group B streptococcal phosphoglycerate kinase.* J Biol Chem, 2012. **287**(34): p. 29035-44.

227. Fulde, M., N. Bernardo-Garcia, M. Rohde, N. Nachtigall, R. Frank, K.T. Preissner, J. Klett, A. Morreale, G.S. Chhatwal, J.A. Hermoso, and S. Bergmann, *Pneumococcal phosphoglycerate kinase interacts with plasminogen and its tissue activator.* Thromb Haemost, 2014. **111**(3): p. 401-16.

228. Blom, A.M., S. Bergmann, M. Fulde, K. Riesbeck, and V. Agarwal, *Streptococcus pneumoniae phosphoglycerate kinase is a novel complement inhibitor affecting the membrane attack complex formation.* J Biol Chem, 2014.

229. Henderson, B. and A. Martin, *Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease.* Infect Immun, 2011. **79**(9): p. 3476-91.

230. LaRock, C.N. and V. Nizet, *Cationic antimicrobial peptide resistance mechanisms of streptococcal pathogens.* Biochim Biophys Acta, 2015. **1848**(11 Pt B): p. 3047-54.

231. Kristian, S.A., V. Datta, C. Weidenmaier, R. Kansal, I. Fedtke, A. Peschel, R.L. Gallo, and V. Nizet, *D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion.* J Bacteriol, 2005. **187**(19): p. 6719-25.

232. van Sorge, N.M., J.N. Cole, K. Kuipers, A. Henningham, R.K. Aziz, A. Kasirer-Friede, L. Lin, E.T. Berends, M.R. Davies, G. Dougan, F. Zhang, S. Dahesh, L. Shaw, J. Gin, M. Cunningham, J.A. Merriman, J. Hutter, B. Lepenies, S.H. Rooijakkers, R. Malley, M.J. Walker, S.J. Shattil, P.M. Schlievert, B. Choudhury, and V. Nizet, *The classical lancefield antigen of group a Streptococcus is a virulence determinant with implications for vaccine design.* Cell Host Microbe, 2014. **15**(6): p. 729-40.

233. Fernie-King, B.A., D.J. Seilly, M.J. Binks, K.S. Sriprakash, and P.J. Lachmann, *Streptococcal DRS (distantly related to SIC) and SIC inhibit antimicrobial peptides, components of mucosal innate immunity: a comparison of their activities.* Microbes Infect, 2007. **9**(3): p. 300-7.

234. Frick, I.M., P. Akesson, M. Rasmussen, A. Schmidtchen, and L. Bjorck, *SIC, a secreted protein of Streptococcus pyogenes that inactivates antibacterial peptides.* J Biol Chem, 2003. **278**(19): p. 16561-6.

235. LaRock, C.N., S. Dohrmann, J. Todd, R. Corriden, J. Olson, T. Johannssen, B. Lepenies, R.L. Gallo, P. Ghosh, and V. Nizet, *Group A Streptococcal M1 Protein Sequesters Cathelicidin to Evade Innate Immune Killing.* Cell Host Microbe, 2015. **18**(4): p. 471-7.

236. Lauth, X., M. von Kockritz-Blickwede, C.W. McNamara, S. Myskowski, A.S. Zinkernagel, B. Beall, P. Ghosh, R.L. Gallo, and V. Nizet, *M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition.* J Innate Immun, 2009. **1**(3): p. 202-14.

237. Hollands, A., D. Gonzalez, E. Leire, C. Donald, R.L. Gallo, M. Sanderson-Smith, P.C. Dorrestein, and V. Nizet, *A bacterial pathogen co-opts host plasmin to resist killing by cathelicidin antimicrobial peptides.* J Biol Chem, 2012. **287**(49): p. 40891-7.

238. Xhindoli, D., S. Pacor, F. Guida, N. Antcheva, and A. Tossi, *Native oligomerization determines the mode of action and biological activities of human cathelicidin LL-37.* Biochem J, 2014. **457**(2): p. 263-75.

239. Kristian, S.A., A.M. Timmer, G.Y. Liu, X. Lauth, N. Sal-Man, Y. Rosenfeld, Y. Shai, R.L. Gallo, and V. Nizet, *Impairment of innate immune killing mechanisms by bacteriostatic antibiotics.* FASEB J, 2007. **21**(4): p. 1107-16.

240. Pathirana, R.D. and M. Kaparakis-Liaskos, *Bacterial membrane vesicles: Biogenesis, immune regulation and pathogenesis.* Cell Microbiol, 2016. **18**(11): p. 1518-1524.

241. Kaparakis-Liaskos, M. and R.L. Ferrero, *Immune modulation by bacterial outer membrane vesicles.* Nat Rev Immunol, 2015. **15**(6): p. 375-87.

242. Kuehn, M.J. and N.C. Kesty, *Bacterial outer membrane vesicles and the host-pathogen interaction.* Genes Dev, 2005. **19**(22): p. 2645-55.

243. Kulp, A. and M.J. Kuehn, *Biological functions and biogenesis of secreted bacterial outer membrane vesicles.* Annu Rev Microbiol, 2010. **64**: p. 163-84.

244. Lee, E.Y., D.Y. Choi, D.K. Kim, J.W. Kim, J.O. Park, S. Kim, S.H. Kim, D.M. Desiderio, Y.K. Kim, K.P. Kim, and Y.S. Gho, *Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of Staphylococcus aureus-derived membrane vesicles.* Proteomics, 2009. **9**(24): p. 5425-36.

245. Rivera, J., R.J. Cordero, A.S. Nakouzi, S. Frases, A. Nicola, and A. Casadevall, *Bacillus anthracis produces membrane-derived vesicles containing biologically active toxins.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 19002-7.

246. Olaya-Abril, A., R. Prados-Rosales, M.J. McConnell, R. Martin-Pena, J.A. Gonzalez-Reyes, I. Jimenez-Munguia, L. Gomez-Gascon, J. Fernandez, J.L. Luque-Garcia, C. Garcia-Lidon, H. Estevez, J. Pachon, I. Obando, A. Casadevall, L.A. Pirofski, and M.J. Rodriguez-Ortega, *Characterization of protective extracellular membrane-derived vesicles produced by Streptococcus pneumoniae.* J Proteomics, 2014. **106**: p. 46-60.

247. Biagini, M., M. Garibaldi, S. Aprea, A. Pezzicoli, F. Doro, M. Becherelli, A.R. Taddei, C. Tani, S. Tavarini, M. Mora, G. Teti, U. D'Oro, S. Nuti, M. Soriani, I. Margarit, R. Rappuoli, G. Grandi, and N. Norais, *The human pathogen Streptococcus pyogenes releases lipoproteins as Lipoprotein-rich Membrane Vesicles.* Mol Cell Proteomics, 2015.

248. Johansson, L., P. Thulin, D.E. Low, and A. Norrby-Teglund, *Getting under the skin: the immunopathogenesis of Streptococcus pyogenes deep tissue infections.* Clin Infect Dis, 2010. **51**(1): p. 58-65.

249. Velarde, J.J., M. Ashbaugh, and M.R. Wessels, *The human antimicrobial peptide LL-37 binds directly to CsrS, a sensor histidine kinase of group A Streptococcus, to activate expression of virulence factors.* J Biol Chem, 2014. **289**(52): p. 36315-24.

250. Resch, U., J.A. Tsatsaronis, A. Le Rhun, G. Stubiger, M. Rohde, S. Kasvandik, S. Holzmeister, P. Tinnefeld, S.N. Wai, and E. Charpentier, *A Two-Component Regulatory System Impacts Extracellular Membrane-Derived Vesicle Production in Group A Streptococcus.* MBio, 2016. **7**(6).

251. Valeva, A., I. Walev, M. Pinkernell, B. Walker, H. Bayley, M. Palmer, and S. Bhakdi, *Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells.* Proc Natl Acad Sci U S A, 1997. **94**(21): p. 11607-11.

252. Vandenesch, F., G. Lina, and T. Henry, *Staphylococcus aureus hemolysins, bi-component leukocidins, and cytolytic peptides: a redundant arsenal of membrane-damaging virulence factors?* Front Cell Infect Microbiol, 2012. **2**: p. 12.

253. Harris, H.E., U. Andersson, and D.S. Pisetsky, *HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease.* Nat Rev Rheumatol, 2012. **8**(4): p. 195-202.

254. Foell, D., H. Wittkowski, T. Vogl, and J. Roth, *S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules.* J Leukoc Biol, 2007. **81**(1): p. 28-37.

255. Yeh, M.C., V. Mukaro, C.S. Hii, and A. Ferrante, *Regulation of neutrophil-mediated killing of Staphylococcus aureus and chemotaxis by c-jun NH2 terminal kinase.* J Leukoc Biol, 2010. **87**(5): p. 925-32.

256. Colin, D.A. and H. Monteil, *Control of the oxidative burst of human neutrophils by staphylococcal leukotoxins.* Infect Immun, 2003. **71**(7): p. 3724-9.

257. Genestier, A.L., M.C. Michallet, G. Prevost, G. Bellot, L. Chalabreysse, S. Peyrol, F. Thivolet, J. Etienne, G. Lina, F.M. Vallette, F. Vandenesch, and L. Genestier, *Staphylococcus aureus Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils.* J Clin Invest, 2005. **115**(11): p. 3117-27.

258. Ma, X., W. Chang, C. Zhang, X. Zhou, and F. Yu, *Staphylococcal Panton-Valentine leukocidin induces pro-inflammatory cytokine production and nuclear factor-kappa B activation in neutrophils.* PLoS One, 2012. **7**(4): p. e34970.

259. Bae, I.G., G.T. Tonthat, M.E. Stryjewski, T.H. Rude, L.F. Reilly, S.L. Barriere, F.C. Genter, G.R. Corey, and V.G. Fowler, Jr., *Presence of genes encoding the panton-valentine leukocidin exotoxin is not the primary determinant of outcome in patients with complicated skin and skin structure infections due to methicillin-resistant Staphylococcus aureus: results of a multinational trial.* J Clin Microbiol, 2009. **47**(12): p. 3952-7.

260. Campbell, S.J., H.S. Deshmukh, C.L. Nelson, I.G. Bae, M.E. Stryjewski, J.J. Federspiel, G.T. Tonthat, T.H. Rude, S.L. Barriere, R. Corey, and V.G. Fowler,

Jr., *Genotypic characteristics of Staphylococcus aureus isolates from a multinational trial of complicated skin and skin structure infections.* J Clin Microbiol, 2008. **46**(2): p. 678-84.

261. Lalani, T., J.J. Federspiel, H.W. Boucher, T.H. Rude, I.G. Bae, M.J. Rybak, G.T. Tonthat, G.R. Corey, M.E. Stryjewski, G. Sakoulas, V.H. Chu, J. Alder, J.N. Steenbergen, S.A. Luperchio, M. Campion, C.W. Woods, and V.G. Fowler, *Associations between the genotypes of Staphylococcus aureus bloodstream isolates and clinical characteristics and outcomes of bacteremic patients.* J Clin Microbiol, 2008. **46**(9): p. 2890-6.

262. Boakes, E., A.M. Kearns, C. Badiou, G. Lina, R.L. Hill, and M.J. Ellington, *Do differences in Panton-Valentine leukocidin production among international methicillin-resistant Staphylococcus aureus clones affect disease presentation and severity?* J Clin Microbiol, 2012. **50**(5): p. 1773-6.

263. Hamilton, S.M., A.E. Bryant, K.C. Carroll, V. Lockary, Y. Ma, E. McIndoo, L.G. Miller, F. Perdreau-Remington, J. Pullman, G.F. Risi, D.B. Salmi, and D.L. Stevens, *In vitro production of panton-valentine leukocidin among strains of methicillin-resistant Staphylococcus aureus causing diverse infections.* Clin Infect Dis, 2007. **45**(12): p. 1550-8.

264. Sharma-Kuinkel, B.K., S.H. Ahn, T.H. Rude, Y. Zhang, S.Y. Tong, F. Ruffin, F.C. Genter, K.R. Braughton, F.R. Deleo, S.L. Barriere, and V.G. Fowler, Jr., *Presence of genes encoding panton-valentine leukocidin is not the primary determinant of outcome in patients with hospital-acquired pneumonia due to Staphylococcus aureus.* J Clin Microbiol, 2012. **50**(3): p. 848-56.